CLAIMS

WHAT IS CLAIMED IS:

1. A eukaryotic cell comprising:

(a) a messenger RNA (mRNA) comprising a codon comprising a first unnatural base, wherein the mRNA encodes a polypeptide comprising at least one unnatural amino acid encoded by the codon; and

(b) a pyrrolysyl or tyrosyl transfer RNA (tRNA) comprising an anticodon comprising a second unnatural base,

(c) a pyrrolysyl or tyrosyl tRNA synthetase, wherein the tRNA synthetase is characterized in that it aminoacylates the tRNA with the at least one unnatural amino acid;

wherein the first and second unnatural bases are capable of forming an unnatural base pair (UBP) in the eukaryotic cell, and wherein the codon and the anticodon each independently comprise three contiguous nucleobases (N-N-N);

wherein:

the first unnatural base (X) is located at the middle position (N-X-N) of the codon, and the second unnatural base (Y) is located at the middle position (N-Y-N) of the anticodon; or

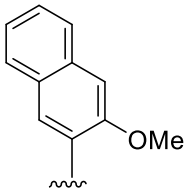
the first unnatural base (X) is located at the last position (N-N-X) of the codon, and the second unnatural base (Y) is located at the first position (Y-N-N) of the anticodon.

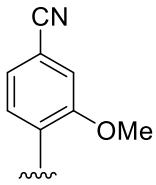
2. The eukaryotic cell of claim 1, wherein the tRNA is charged with the at least one unnatural amino acid.

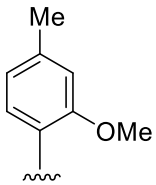
3. The eukaryotic cell of claim 1 or claim 2, further comprising a polypeptide translated from the mRNA, wherein the polypeptide comprises the at least one unnatural amino acid.

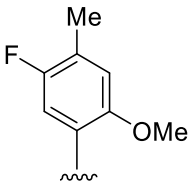
4. The eukaryotic cell of any one of the preceding claims, wherein the polypeptide comprises a eukaryotic glycosylation pattern.

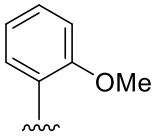
5. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base and the second unnatural base are each, independently, selected from:

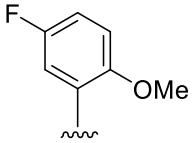


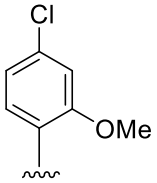
(NaM), 

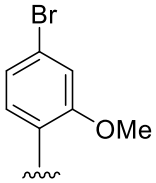
(CNMO), 

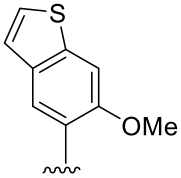
(MMO2), 

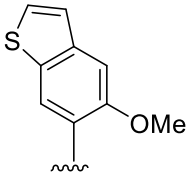
(5FM), 

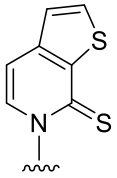
(2OMe), 

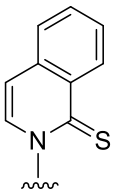
(5F2OMe), 

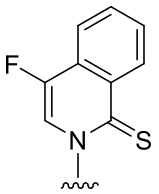
(ClMO), 

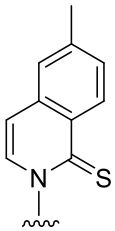
(BrMO), 

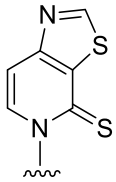
(PTMO), 

(MTMO), 

(TPT3), 

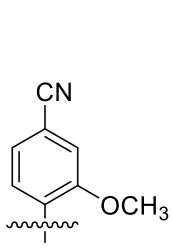
(SICS), 

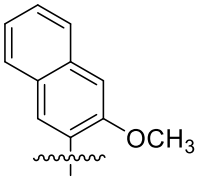
(FSICS), 

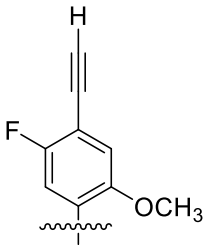
(5SICS), and 

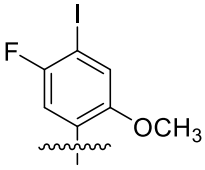
(TAT1), wherein the wavy line indicates a bond to a ribosyl moiety.

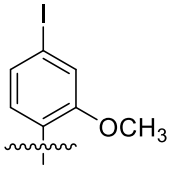
6. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base is selected from:

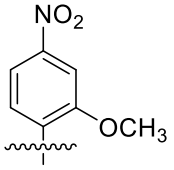


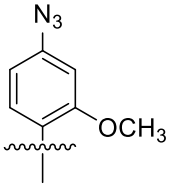
, 

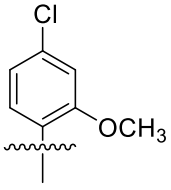
, 

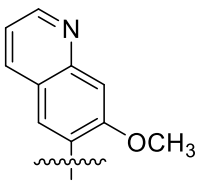
, 

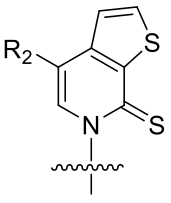
, 

, 

, 

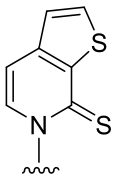
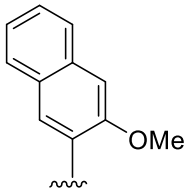
, 

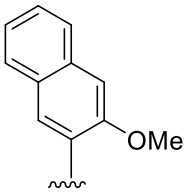
, 

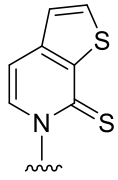
or 

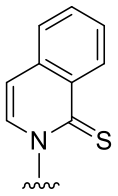
, wherein R_2 is selected from: hydrogen, alkyl, alkenyl, alkynyl, methoxy, methanethiol, methaneseleno, halogen, cyano, and azido, and the wavy line indicates a bond to a ribosyl moiety.

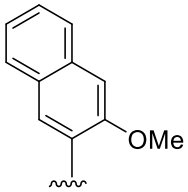
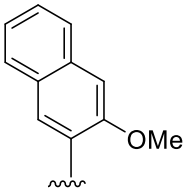
7. The eukaryotic cell of any one of the preceding claims, (a)(i) when the first

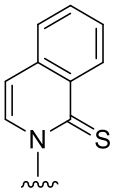
unnatural base is  (TPT3), the second unnatural base is  (NaM), or

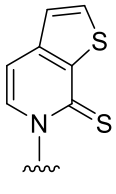
(ii) when the first unnatural base is  (NaM), the second unnatural base is

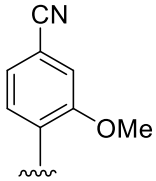
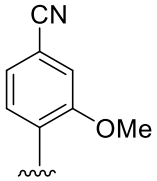
 (TPT3), wherein the wavy line indicates a bond to a ribosyl moiety;

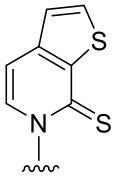
or (b)(i) when the first unnatural base is  (5SICS), the second unnatural base is

 (NaM), or (ii) when the first unnatural base is  (NaM), the second

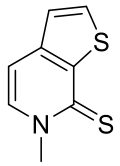
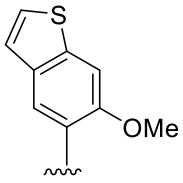
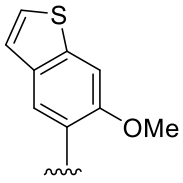
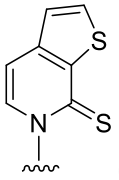
unnatural base is  (5SICS), wherein the wavy line indicates a bond to a ribosyl moiety;

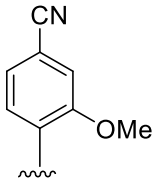
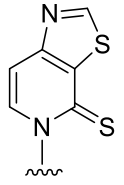
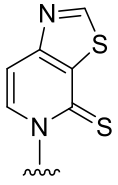
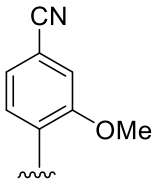
or (c)(i) when the first unnatural base is  (TPT3) and the second unnatural base is

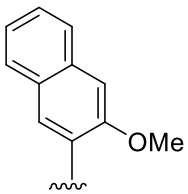
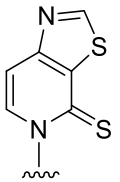
 (CNMO), or (ii) when the first unnatural base is  (CNMO), the

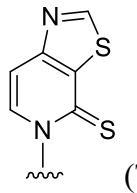
second unnatural base is  (TPT3), wherein the wavy line indicates a bond to a

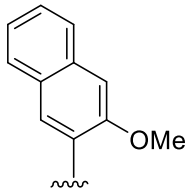
ribosyl moiety;

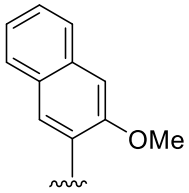
or (d)(i) when the first unnatural base is  (TPT3), the second unnatural base is  (PTMO), or (ii) when the first unnatural base is  (PTMO), the second unnatural base is  (TPT3), wherein the wavy line indicates a bond to a ribosyl moiety;

or (e)(i) when the first unnatural base is  (CNMO), the second unnatural base is  (TAT1), or (ii) when the first unnatural base is  (TAT1), the second unnatural base is  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety;

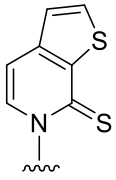
or (f)(i) when the first unnatural base is  (NaM), the second unnatural base is  (TAT1), or (ii) when the first unnatural base is

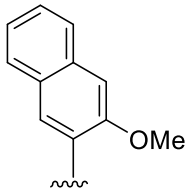


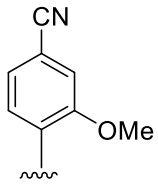
(TAT1), the second unnatural base is 

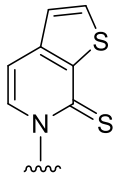
(NaM), wherein the wavy line indicates a bond to a ribosyl moiety; or (g) wherein the first unnatural base and the second unnatural base are each 

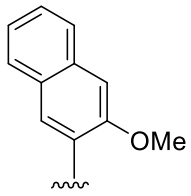
(NaM), wherein the wavy line indicates a bond to a ribosyl moiety.

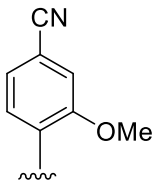
8. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base (X) is located at the middle position (N-X-N) in the codon and the first unnatural base is selected from 

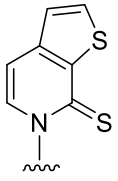
(TPT3), 

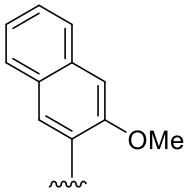
(NaM), and 

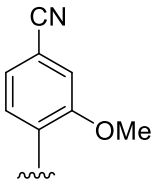
(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the first unnatural base (X) is located at the last position (N-N-X) in the codon and the first unnatural base is selected from 

(TPT3), 

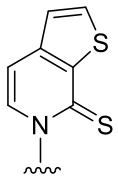
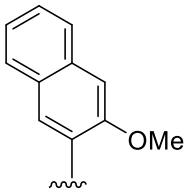
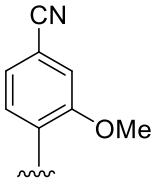
(NaM), and 

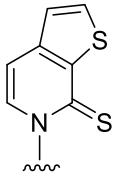
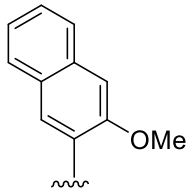
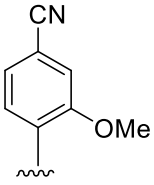
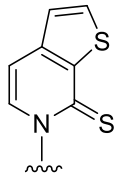
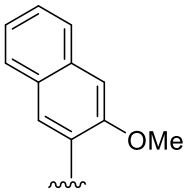
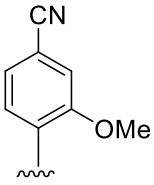
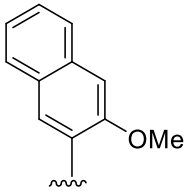
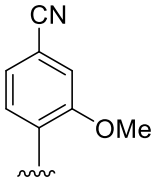
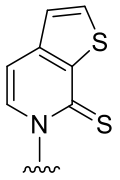
(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the second unnatural base (Y) is located at the first position (Y-N-N) of the anticodon, and the second unnatural base is selected from 

(TPT3), 

(NaM), and 

(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the second unnatural base (Y) is located at the middle position (N-Y-

N) of the anticodon, and the second unnatural base is selected from  (TPT3),  (NaM), and  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety.

9. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base (X) and the second unnatural base (Y) are each independently selected from:  (TPT3),  (NaM), and  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the first unnatural base (X) and the second unnatural base (Y) are both  (TPT3),  (NaM), or  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the first unnatural base (X) is selected from  (NaM) and  (CNMO), and the second unnatural base (Y) is  (TPT3), wherein in each case the wavy line indicates a bond to a ribosyl moiety.

10. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base (X) and the second unnatural base (Y) are the same.

11. The eukaryotic cell of any one of claims 1-9, wherein the first unnatural base (X) and the second unnatural base (Y) are different.

12. The eukaryotic cell of any one of the preceding claims, wherein the three contiguous nucleobases in the codon is selected from AXC, GXC, and GXU, wherein X is the first unnatural base.

13. The eukaryotic cell of claim 12, wherein the three contiguous nucleobases in the anticodon is selected from GYU, GYC, and AYC, wherein Y is the second unnatural base.

14. The eukaryotic cell of claim 12, wherein the three contiguous nucleobases in the codon is AXC and the three contiguous nucleobases in the anticodon is GYU; or the three contiguous nucleobases in the codon is GXC and the three contiguous nucleobases in the anticodon is GYC; or the three contiguous nucleobases in the codon is GXU and the three contiguous nucleobases in the anticodon is AYC.

15. The eukaryotic cell of any one of the preceding claims, wherein the first unnatural base or the second unnatural base comprises a modified sugar moiety independently selected from:
a 2' modification comprising:
OH, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2F, or a combination thereof;
O-alkyl, S-alkyl, N-alkyl, or a combination thereof;
O-alkenyl, S-alkenyl, N-alkenyl, or a combination thereof;
O-alkynyl, S-alkynyl, N-alkynyl, or a combination thereof;
O-alkyl-O-alkyl, 2'-F, 2'-OCH3, 2'-$O(CH_2)_2OCH_3$, or a combination thereof, wherein the alkyl, alkenyl and alkynyl is substituted or unsubstituted C_1-C_{10}, alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, -$O[(CH_2)_nO]_mCH_3$, -$O(CH_2)_nOCH_3$, -$O(CH_2)_nNH_2$, -$O(CH_2)_nCH_3$, -$O(CH_2)_n$-NH_2, or -$O(CH_2)_nON[(CH_2)_nCH_3)]_2$, wherein n and m are from 1 to 10;
a 5' modification comprising:
5'-vinyl, 5'-methyl (R or S), or a combination thereof;
a 4' modification comprising:
4'-S, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving pharmacokinetic properties of an oligonucleotide, or a group for improving pharmacodynamic properties of an oligonucleotide, or a combination thereof;
or a combination thereof.

16. The eukaryotic cell of any one of claims 1 to 15, wherein the at least one

unnatural amino acid:

is a lysine analogue;

comprises an aromatic side chain;

comprises an azido group;

comprises an alkyne group; or

comprises an aldehyde or ketone group.

17. The eukaryotic cell of claim 16, wherein the at least one unnatural amino acid comprises N6-((azidoethoxy)-carbonyl)-L-lysine (AzK).

18. The eukaryotic cell of any one of claims 1 to 17, wherein the eukaryotic cell is a human cell.

19. The eukaryotic cell of any one of claims 1 to 18, wherein the eukaryotic cell is a mammalian cell.

20. The eukaryotic cell of any one of claims 1-17, wherein the eukaryotic cell is a HEK293T cell or a Chinese hamster ovary (CHO) cell.

21. The eukaryotic cell of any one of claims 1-20, further comprising a polypeptide translated from the mRNA, wherein the polypeptide comprises the at least one unnatural amino acid and a mammalian glycosylation pattern.

22. The eukaryotic cell of any one of claims 1-21, wherein the eukaryotic cell is an isolated.

23. The eukaryotic cell of any one of claims 1-22, wherein the pyrrolysyl or tyrosyl tRNA is from *Methanococcus jannaschii*, *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*, and/or the pyrrolysyl or tyrosyl tRNA synthetase is from *Methanococcus jannaschii*, *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*.

24. A semi-synthetic organism comprising the eukaryotic cell of any one of claims 1-23, wherein the eukaryotic cell is a non-human eukaryotic cell.

25. A eukaryotic cell culture comprising a plurality of eukaryotic cells of any one of claims 1-23.

26. The cell of any one of claims 1-23 for use in therapy, comprising contacting an organism with the eukaryotic cell.

27. The cell for use of claim 26, wherein the organism is a mammal.

28. The cell for use of claim 27, wherein the mammal is a human.

29. A method of producing a polypeptide comprising at least one unnatural amino acid in an isolated eukaryotic cell, comprising:

introducing into an eukaryotic cell:

(i) a messenger RNA (mRNA) comprising a codon comprising a first unnatural base, wherein the mRNA encodes a polypeptide comprising at least one unnatural amino acid encoded by the codon; and

(ii) a pyrrolysyl or tyrosyl transfer RNA (tRNA) comprising an anticodon comprising a second unnatural base, wherein the first and second unnatural bases are capable of forming an unnatural base pair (UBP) in the eukaryotic cell, and wherein the codon and the anticodon each independently comprise three contiguous nucleobases (N-N-N);

wherein the eukaryotic cell comprises an expression vector encoding a pyrrolysyl or tyrosyl tRNA synthetase, wherein the tRNA synthetase is characterized in that it aminoacylates the tRNA with the at least one unnatural amino acid;

wherein

the first unnatural base (X) is located at the middle position (N-X-N) of the codon, and the second unnatural base (Y) is located at the middle position (N-Y-N) of the anticodon; or

the first unnatural base (X) is located at the last position (N-N-X) of the codon, and the second unnatural base (Y) is located at the first position (Y-N-N) of the anticodon; and

wherein the eukaryotic cell is capable of translating the mRNA into the polypeptide comprising the at least one unnatural amino acid from the mRNA using the tRNA.

30. The method of the preceding claim, wherein the tRNA is charged with an unnatural amino acid.

31. A method of producing a polypeptide comprising at least one unnatural amino acid in an isolated eukaryotic cell, comprising:

culturing the eukaryotic cell of any one of claims 1-23 under conditions such that the mRNA is translated in the eukaryotic cell to a polypeptide comprising the at least one unnatural amino acid using the tRNA and a ribosome that is endogenous to the eukaryotic cell.

32. A method of producing a polypeptide in an isolated eukaryotic cell, wherein the polypeptide comprises at least one unnatural amino acid, the method comprising:

contacting the eukaryotic cell of any one of claims 1-23 with the at least one unnatural amino acid under conditions such that the mRNA is translated in the eukaryotic cell to a polypeptide comprising the at least one unnatural amino acid.

33. A system for producing an unnatural polypeptide comprising:

(a) at least one unnatural amino acid;

(b) an mRNA encoding an unnatural polypeptide comprising the at least one unnatural amino acid, said mRNA comprising a codon comprising a first unnatural bases;

(c) a pyrrolysyl or tyrosyl tRNA comprising an anti-codon comprising a second unnatural base; and

(d) a eukaryotic ribosome capable of translating the mRNA into a polypeptide comprising the at least one unnatural amino acid using the pyrrolysyl or tyrosyl tRNA

wherein (i) the pyrrolysyl or tyrosyl tRNA is charged with the at least one unnatural amino acid, or (ii) the system further comprises a pyrrolysyl or tyrosyl tRNA synthetase or one or more nucleic acid constructs comprising a nucleic acid sequence encoding a pyrrolysyl or tyrosyl tRNA synthetase, wherein the pyrrolysyl or tyrosyl tRNA synthetase is characterized in that it aminoacylates the tRNA with the at least one unnatural amino acid;

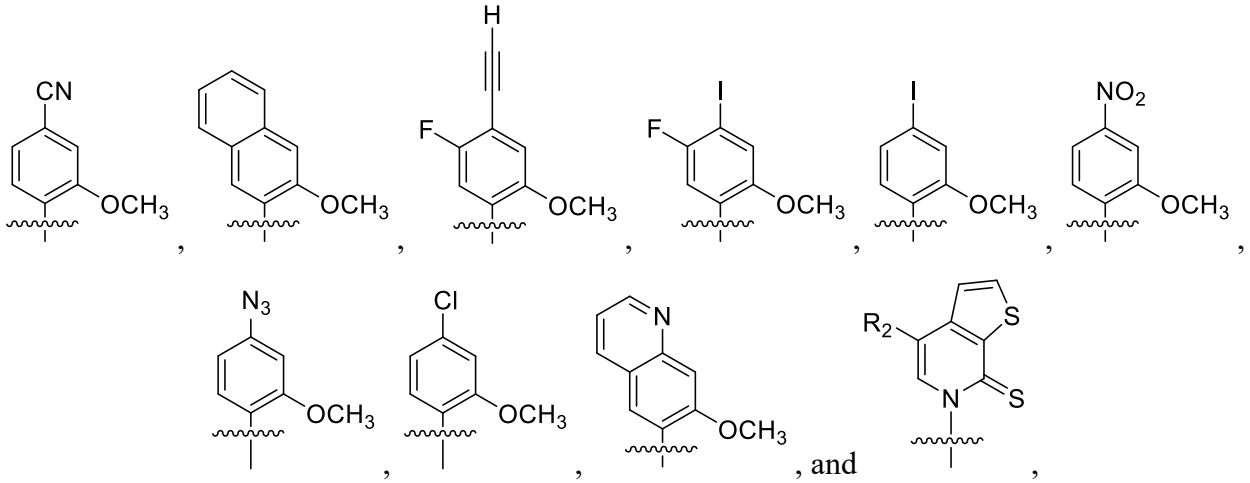
wherein the first unnatural base and second unnatural base are capable of forming an unnatural base pair (UBP), and wherein the codon and the anticodon each independently comprise three contiguous nucleobases (N-N-N);

wherein:

the first unnatural base (X) is located at the middle position (N-X-N) of the codon, and the second unnatural base (Y) is located at the middle position (N-Y-N) of the anticodon; or

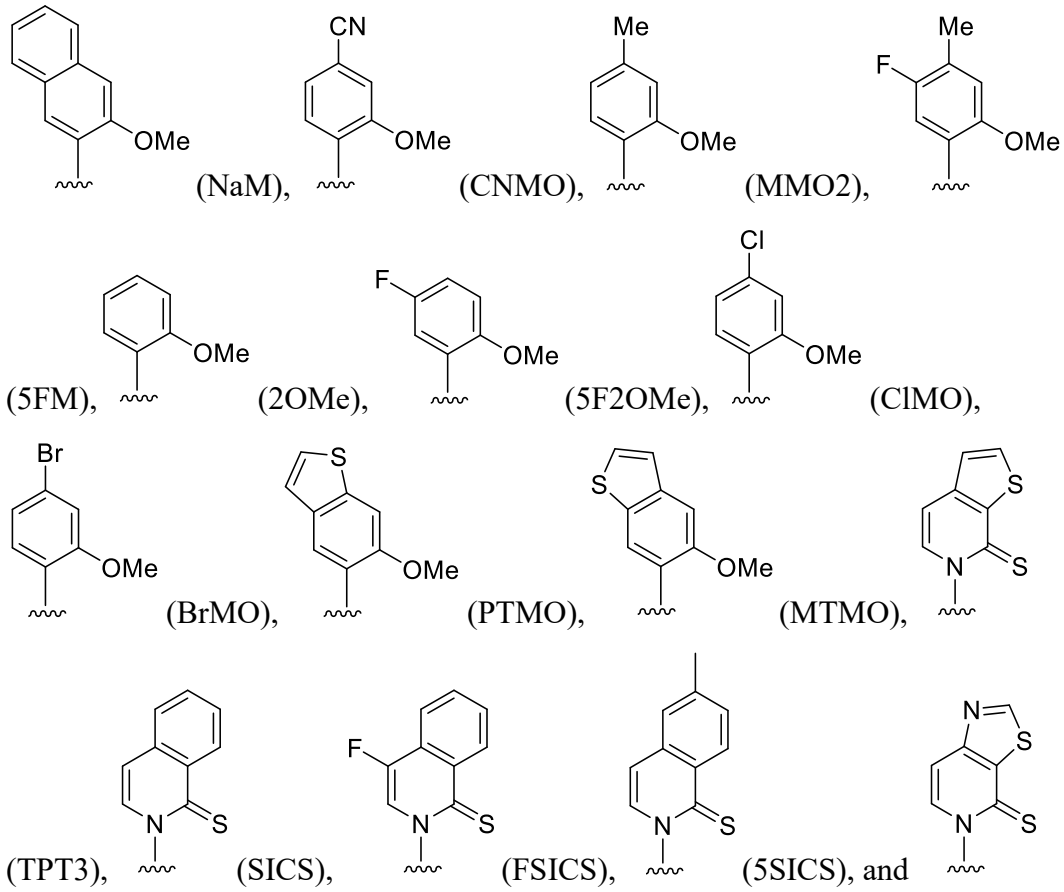
the first unnatural base (X) is located at the last position (N-N-X) of the codon, and the second unnatural base (Y) is located at the first position (Y-N-N) of the anticodon.

34. The system of claim 33, wherein the first unnatural base and the second unnatural base are each, independently, selected from:

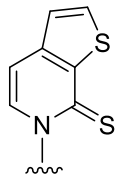


wherein R_2 is selected from: hydrogen, alkyl, alkenyl, alkynyl, methoxy, methanethiol, methaneseleno, halogen, cyano, and azido, and the wavy line indicates a bond to a ribosyl moiety.

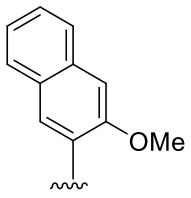
35. The system of claim 33 or claim 34, wherein the first unnatural base or the second unnatural base is independently selected from:



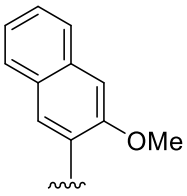
(NaM), (CNMO), (MMO2), (5FM), (2OMe), (5F2OMe), (ClMO), (BrMO), (PTMO), (MTMO), (TPT3), (SICS), (FSICS), (5SICS), and (TAT1), wherein the wavy line indicates a bond to a ribosyl moiety.

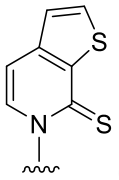
36. The system of claim 35, wherein (a)(i) when the first unnatural base is 

(TPT3),

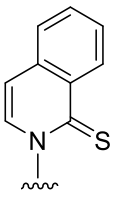


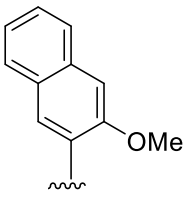
the second unnatural base is (NaM), or (ii) when the first unnatural base is

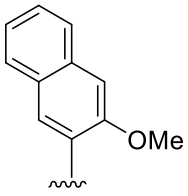


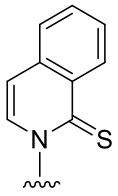
(NaM), the second unnatural base is 

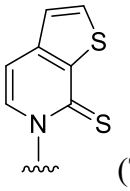
(TPT3), wherein the wavy line indicates a bond to a ribosyl moiety; or (b)(i) when the first unnatural base is



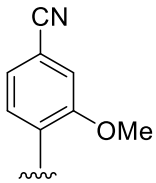
(5SICS), the second unnatural base is 

(NaM), or (ii) when the first unnatural base is 

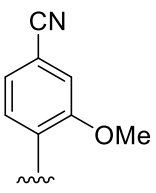
(NaM), the second unnatural base is 

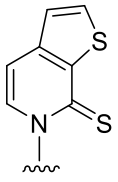
(5SICS), wherein the wavy line indicates a bond to a ribosyl moiety; or (c)(i) when the first unnatural base is 

(TPT3),

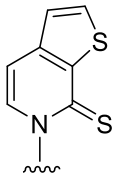


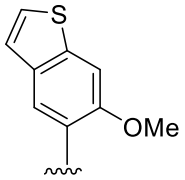
the second unnatural base is (CNMO), or (ii) when the first unnatural base is

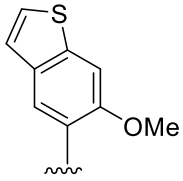


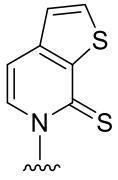
(CNMO), the second unnatural base is 

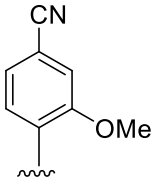
(TPT3), wherein the wavy line indicates a bond to a ribosyl moiety; or (d)(i) when the first unnatural base is

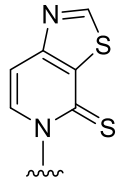


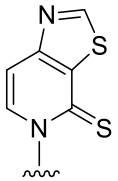
(TPT3), the second unnatural base is 

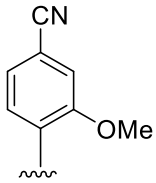
(PTMO), or (ii) when the first unnatural base is 

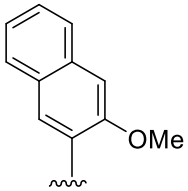
(PTMO), the second unnatural base is 

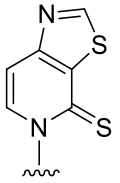
(TPT3), wherein the wavy line indicates a bond to a ribosyl moiety; or (e)(i) when the first unnatural base is 

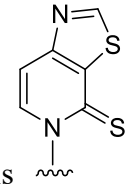
(CNMO), the second unnatural base is 

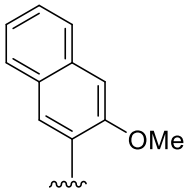
(TAT1), or (ii) when the first unnatural base is 

(TAT1), the second unnatural base is 

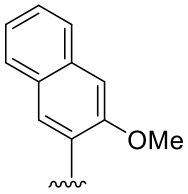
(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or (f)(i) when the first unnatural base is 

(NaM), the second unnatural base is 

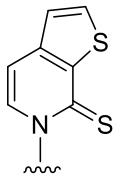
(TAT1), or (ii) when the first unnatural base is 

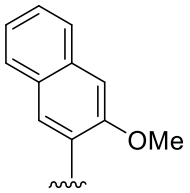
(TAT1), the second unnatural base is 

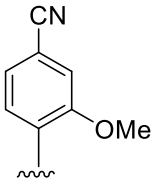
(NaM), wherein the wavy line indicates a bond to a ribosyl moiety; or (g) wherein the first unnatural base and the second unnatural base are

each 

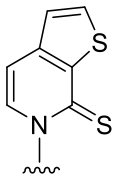
(NaM), wherein the wavy line indicates a bond to a ribosyl moiety; or

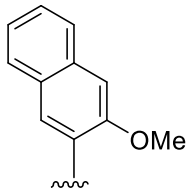
(h) wherein the first unnatural base is selected from 

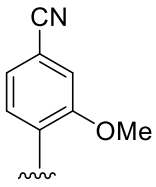
(TPT3), 

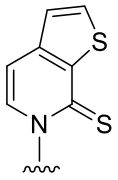
(NaM), and 

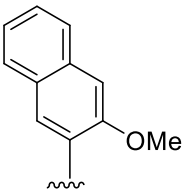
(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety.

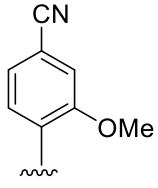
37. The system of claim 33, wherein first unnatural base (**X**) is located at the middle position (N-**X**-N) in the codon, and the first unnatural base is selected from 

(TPT3), 

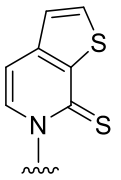
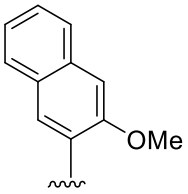
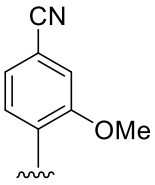
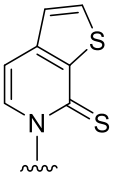
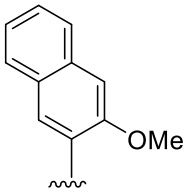
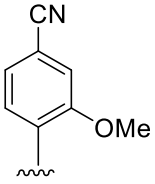
(NaM), and 

(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the first unnatural base (**X**) is located at the last position (N-N-X) in the codon, and the first unnatural base is selected from 

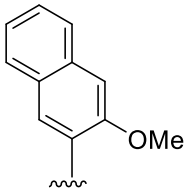
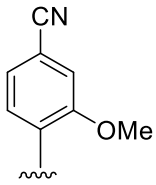
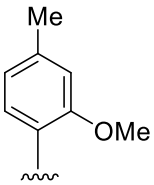
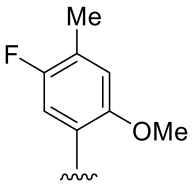
(TPT3), 

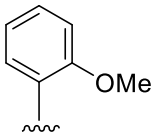
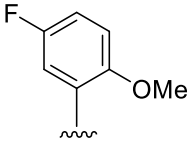
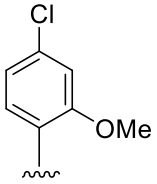
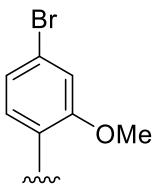
(NaM), 

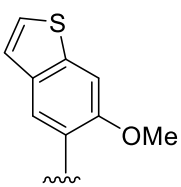
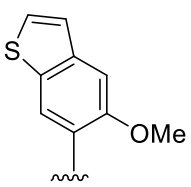
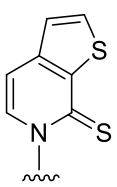
and (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the second unnatural base (**Y**) is located at the first position (**Y**-N-N) of the anticodon,

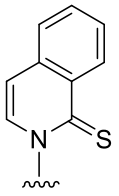
and the second unnatural base is selected from  (TPT3),  (NaM), and  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety; or wherein the second unnatural base (**Y**) is located at the middle position (N-**Y**-N) of the anticodon, and the second unnatural base is selected from  (TPT3),  (NaM), and  (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety.

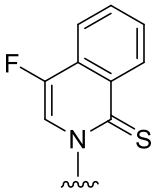
38. The system of claim 33, wherein the first unnatural base (X) and the second unnatural base (Y) are the same and/or the first unnatural base (X) and the second unnatural base (Y) are each selected from:

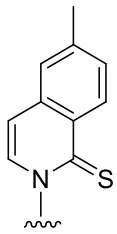
 (NaM),  (CNMO),  (MMO2), 

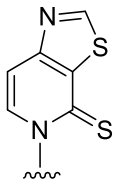
 (5FM),  (2OMe),  (5F2OMe),  (ClMO),

 (BrMO),  (PTMO),  (MTMO),

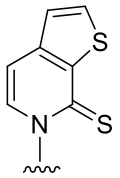


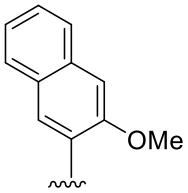
(TPT3), 

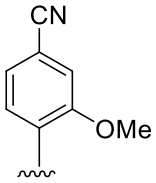
(SICS), 

(FSICS), 

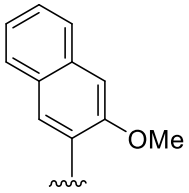
(5SICS), and (TAT1), wherein the wavy line indicates a bond to a ribosyl moiety.

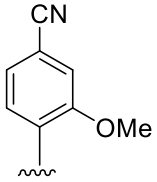
39. The system of claim 38, wherein the first unnatural base (X) and the second unnatural base (Y) are each selected from: 

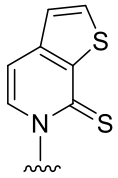
(TPT3), 

(NaM), and 

(CNMO), wherein the wavy line indicates a bond to a ribosyl moiety.

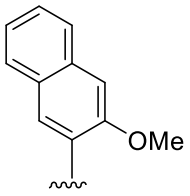
40. The system of claim 39, wherein the first unnatural base (X) is selected from 

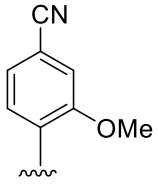
(NaM) and 

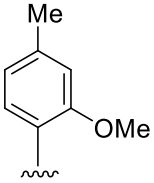
(CNMO), and the second unnatural base (Y) is 

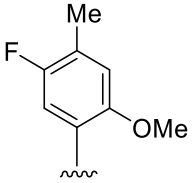
(TPT3), wherein in each case the wavy line indicates a bond to a ribosyl moiety.

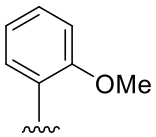
41. The system of claim 33, wherein the first unnatural base (X) and the second unnatural base (Y) are different and/or the first unnatural base (X) and the second unnatural base (Y) are each independently selected from:

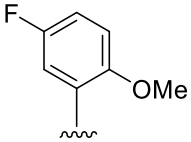


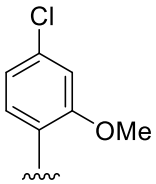
(NaM), 

(CNMO), 

(MMO2), 

(5FM), 

(2OMe), 

(5F2OMe), 

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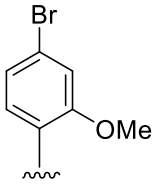
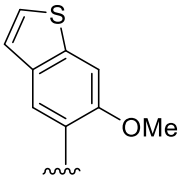
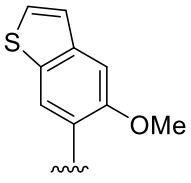
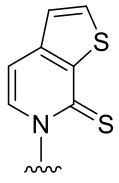
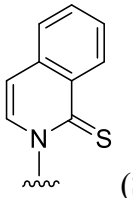
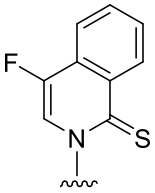


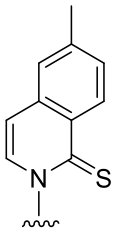
(BrMO), 

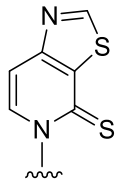
(PTMO), 

(MTMO), 

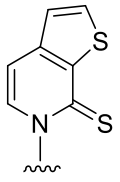
(TPT3), 

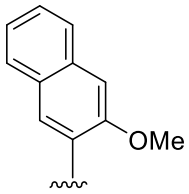
(SICS), 

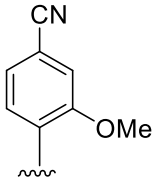
(FSICS), 

(5SICS), and 

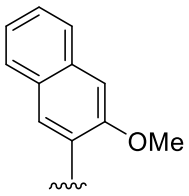
(TAT1), wherein the wavy line indicates a bond to a ribosyl moiety.

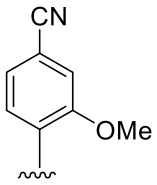
42. The system of claim 41, wherein the first unnatural base (X) and the second unnatural base (Y) are each independently selected from: 

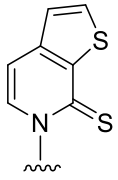
(TPT3), 



(NaM), and (CNMO), wherein the wavy line indicates a bond to a ribosyl moiety.

43. The system of claim 42, wherein the first unnatural base (X) is selected from 

(NaM) and 

(CNMO), and the second unnatural base (Y) is 

(TPT3), wherein in each case the wavy line indicates a bond to a ribosyl moiety.

44. The system of any one of claims 33-43, wherein the three contiguous nucleobases in the codon is selected from AXC, GXC or GXU, wherein X is the first unnatural base.

45. The system of claim 44, wherein the three contiguous nucleobases in the anticodon is selected from GYU, GYC, and AYC, wherein Y is the second unnatural base.

46. The system of claim 45, wherein the codon is AXC and the anticodon is GYU, the codon is GXC and the anticodon is GYC, or the codon is GXU and the anticodon is AYC.

47. The system of any one of claims 33-46, wherein the pyrrolysyl or tyrosyl tRNA is from *Methanococcus jannaschii*, *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*; the pyrrolysyl or tyrosyl tRNA synthetase is from *Methanococcus jannaschii*, *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*.

48. The system of any one of claims 33-47, wherein the system is in vitro or cell-free; the system comprises a cell lysate; and/or the system is a reconstituted system of purified components.

49. The system of any one of claims 33-47, wherein the system is in a eukaryotic cell.

50. The system of any one of claims 33-49, wherein the at least one unnatural amino acid:

is a lysine analogue;
comprises an aromatic side chain;
comprises an azido group;
comprises an alkyne group; or
comprises an aldehyde or ketone group.

51. The system of claim 50, wherein the at least one unnatural amino acid comprises N6-((azidoethoxy)-carbonyl)-L-lysine (AzK).

52. The system of any one of claims 33-51, wherein the pyrrolysyl or tyrosyl tRNA is charged with the at least one unnatural amino acid.