

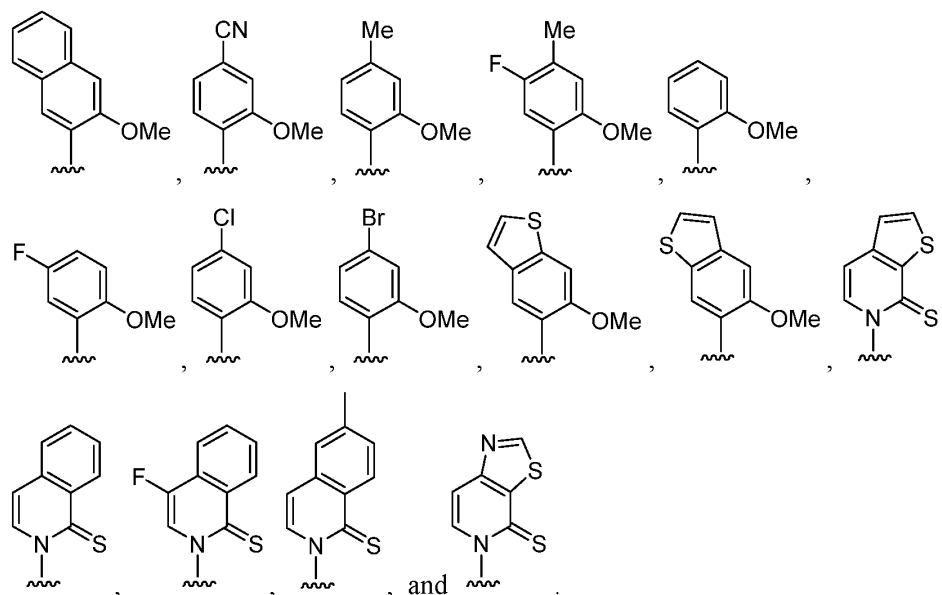
CLAIMS

WHAT IS CLAIMED IS:

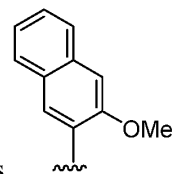
1. A method of synthesizing an unnatural polypeptide comprising:
 - a. providing at least one unnatural deoxyribonucleic acid (DNA) molecule comprising at least four unnatural base pairs, wherein the at least one unnatural DNA molecule encodes (i) a messenger ribonucleic acid (mRNA) molecule comprising at least first and second unnatural codons and (ii) at least first and second transfer RNA (tRNA) molecules, the first tRNA molecule comprising a first unnatural anticodon and the second tRNA molecule comprising a second unnatural anticodon, and the at least four unnatural base pairs in the at least one DNA molecule are in sequence contexts such that the first and second unnatural codons of the mRNA molecule are complementary to the first and second unnatural anticodons, respectively;
 - b. transcribing the at least one unnatural DNA molecule to afford the mRNA;
 - c. transcribing the at least one unnatural DNA molecule to afford the at least first and second tRNA molecules; and
 - d. synthesizing the unnatural polypeptide by translating the unnatural mRNA molecule utilizing the at least first and second unnatural tRNA molecules, wherein each of the at least first and second unnatural anticodons direct site-specific incorporation of an unnatural amino acid into the unnatural polypeptide.
2. The method of claim 1, wherein the at least two unnatural codons each comprise a first unnatural nucleotide positioned at a first position, a second position, or a third position of the codon, optionally wherein the first unnatural nucleotide is positioned at a second position or a third position of the codon.
3. The method of any one of the preceding claims, wherein the at least two unnatural codons each comprises a nucleic acid sequence NNX or NXN, and the unnatural anticodon comprises a nucleic acid sequence XNN, YNN, NXN, or NYN, to form the unnatural codon-anticodon pair comprising NNX-XNN, NNX-YNN, or NXN-NYN, wherein N is any natural nucleotide, X is a first unnatural nucleotide, and Y is a second unnatural nucleotide different from the first unnatural nucleotide, with X-Y forming the unnatural base pair in DNA.

4. The method of claim 3, wherein the codon comprises at least one G or C and the anticodon comprises at least one complementary C or G.
5. The method of claim 3 or 4, wherein X and Y are independently selected from the group consisting of:
 - (i) 2-thiouracil, 2'-deoxyuridine, 4-thio-uracil, uracil-5-yl, hypoxanthin-9-yl (I), 5-halouracil; 5-propynyl-uracil, 6-azo-uracil, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, pseudouracil, uracil-5-oxacetic acid methylester, uracil-5-oxacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, 5-methyl-2-thiouracil, 4-thiouracil, 5-methyluracil, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, or dihydrouracil;
 - (ii) 5-hydroxymethyl cytosine, 5-trifluoromethyl cytosine, 5-halocytosine, 5-propynyl cytosine, 5-hydroxycytosine, cyclocytosine, cytosine arabinoside, 5,6-dihydrocytosine, 5-nitrocytosine, 6-azo cytosine, azacytosine, N4-ethylcytosine, 3-methylcytosine, 5-methylcytosine, 4-acetylcytosine, 2-thiocytosine, phenoxazine cytidine([5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), phenoxazine cytidine (9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), or pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo [2,3-d]pyrimidin-2-one);
 - (iii) 2-aminoadenine, 2-propyl adenine, 2-amino-adenine, 2-F-adenine, 2-amino-propyl-adenine, 2-amino-2'-deoxyadenosine, 3-deazaadenine, 7-methyladenine, 7-deaza-adenine, 8-azaadenine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted adenines, N6-isopentenyladenine, 2-methyladenine, 2,6-diaminopurine, 2-methylthio-N6- isopentenyladenine, or 6-aza-adenine;
 - (iv) 2-methylguanine, 2-propyl and alkyl derivatives of guanine, 3-deazaguanine, 6-thio-guanine, 7-methylguanine, 7-deazaguanine, 7-deazaguanosine, 7-deaza-8-azaguanine, 8-azaguanine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted guanines, 1-methylguanine, 2,2-dimethylguanine, 7-methylguanine, or 6-aza-guanine; and
 - (v) hypoxanthine, xanthine, 1-methylinosine, queosine, beta-D-galactosylqueosine, inosine, beta-D-mannosylqueosine, wybutoxosine, hydroxyurea, (acp3)w, 2-aminopyridine, or 2-pyridone.

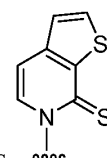
6. The method of claim 4 or 5, wherein the bases comprising each of X and Y are independently selected from the group consisting of:



7. The method of claim 6, wherein the base comprising each X is



8. The method of claim 6 or 7, wherein the base comprising each Y is



9. The method of any one of claims 3-8, wherein NNX-XNN is selected from the group consisting of UUX-XAA, UGX-XCA, CGX-XCG, AGX-XCU, GAX-XUC, CAX-XUG, AUX-XAU, CUX-XAG, GUX-XAC, UAX-XUA, and GGX-XCC.
10. The method of any one of claims 3-8, wherein NNX-YNN is selected from the group consisting of UUX-YAA, UGX-YCA, CGX-YCG, AGX-YCU, GAX-YUC, CAX-YUG, AUX-YAU, CUX-YAG, GUX-YAC, UAX-YUA, and GGX-YCC.

11. The method of any one of claims 3-8, wherein NXN-NYN is selected from the group consisting of GXU-AYC, CXU-AYG, GXG-CYC, AXG-CYU, GXC-GYC, AXC-GYU, GXA-UYC, CXC-GYG, and UXC-GYA.
12. The method of any one of the preceding claims, wherein the at least two unnatural tRNA molecules each comprise a different unnatural anticodon.
13. The method of claim 12, wherein the at least two unnatural tRNA molecules comprise a pyrrollysyl tRNA from the *Methanosarcina* genus and the tyrosyl tRNA from *Methanocaldococcus jannaschii*, or derivatives thereof.
14. The method of any one of claims 11-13, comprising charging the at least two unnatural tRNA molecules by an amino-acyl tRNA synthetase.
15. The method of claim 14, wherein the tRNA synthetase is selected from a group consisting of chimeric PylRS (chPylRS) and *M. jannaschii* AzFRS (*MjpAzFRS*).
16. The method of claim 12 or 13, comprising charging the at least two unnatural tRNA molecules by at least two different tRNA synthetases.
17. The method of claim 16, wherein the at least two different tRNA synthetases comprise chimeric PylRS (chPylRS) and *M. jannaschii* AzFRS (*MjpAzFRS*).
18. The method of any one of claims 1-17, wherein the unnatural polypeptide comprises two, three, or more unnatural amino acids.
19. The method of any one of claims 1-18, wherein the unnatural polypeptide comprises at least two unnatural amino acids that are the same.
20. The method of any one of claims 1-18, wherein the unnatural polypeptide comprises at least two different unnatural amino acids.
21. The method of any one of claims 1-20, wherein the unnatural amino acid comprises
 - a lysine analogue;
 - an aromatic side chain;

an azido group;
 an alkyne group; or
 an aldehyde or ketone group.

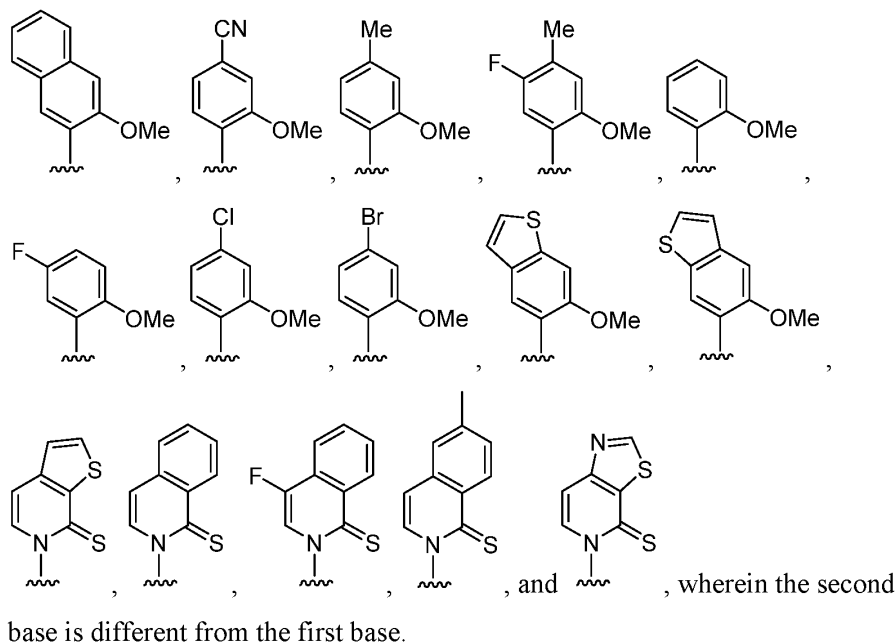
22. The method of any one of the claims 1-20, wherein the unnatural amino acid does not comprise an aromatic side chain.
23. The method of any one of claims 1-20, wherein the unnatural amino acid is selected from N6-azidoethoxy-carbonyl-L-lysine (AzK), N6-propargylethoxy-carbonyl-L-lysine (PraK), N6-(propargyloxy)-carbonyl-L-lysine (PrK), p-azido-phenylalanine (*p*AzF), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, 2-amino-8-oxononanoic acid, 2-amino-8-oxooctanoic acid, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, 2-amino-8-oxononanoic acid, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphonotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, selenocysteine, N6-(((2-azidobenzyl)oxy)carbonyl)-L-lysine, N6-(((3-azidobenzyl)oxy)carbonyl)-L-lysine, and N6-(((4-azidobenzyl)oxy)carbonyl)-L-lysine.
24. The method of any one of the preceding claims, wherein the at least one unnatural DNA molecule is in the form of a plasmid.
25. The method of any one of claims 1-23, wherein the at least one unnatural DNA molecule is integrated into the genome of a cell.
26. The method of claim 24 or 25, wherein the at least one unnatural DNA molecule encodes the unnatural polypeptide.

27. The method of any one of the preceding claims, wherein the method comprises the *in vivo* replication and transcription of the unnatural DNA molecule and the *in vivo* translation of the transcribed mRNA molecule in a cellular organism.
28. The method of claim 27, wherein the cellular organism is a microorganism.
29. The method of claim 28, wherein the cellular organism is a prokaryote.
30. The method of claim 29, wherein the cellular organism is a bacterium.
31. The method of claim 30, wherein the cellular organism is a gram-positive bacterium.
32. The method of claim 30, wherein the cellular organism is a gram-negative bacterium.
33. The method of claim 32, wherein the cellular organism is *Escherichia coli*.
34. The method of any one of the preceding claims, wherein the at least two unnatural base pairs comprise base pairs selected from dCNMO-dTPT3, dNaM-dTPT3, dCNMO-dTAT1, or dNaM-dTAT1.
35. The method of any one of claims 27-34, wherein the cellular organism comprises a nucleoside triphosphate transporter.
36. The method of claim 35, wherein the nucleoside triphosphate transporter comprises the amino acid sequence of *PtNTT2*.
37. The method of claim 36, wherein the nucleoside triphosphate transporter comprises a truncated amino acid sequence of *PtNTT2*, optionally wherein the truncated amino acid sequence of *PtNTT2* is at least 80% identical to a *PtNTT2* encoded by SEQ ID NO.1.
38. The method of any one of claims 27-37, wherein the cellular organism comprises the at least one unnatural DNA molecule.
39. The method of claim 38, wherein the at least one unnatural DNA molecule comprises at least one plasmid.

40. The method of claim 38, wherein the at least one unnatural DNA molecule is integrated into the genome of the cell.
41. The method of claim 39 or 40, wherein the at least one unnatural DNA molecule encodes the unnatural polypeptide.
42. The method of any one of claims 1-24, wherein the method is an *in vitro* method, comprising synthesizing the unnatural polypeptide with a cell-free system.
43. The method of any one of the preceding claims, wherein the unnatural base pairs comprise at least one unnatural nucleotide comprising an unnatural sugar moiety.
44. The method of claim 43, wherein the unnatural sugar moiety comprises a moiety selected from the group consisting of:
a modification at the 2' position comprising:
OH, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, or NH₂F;
O-alkyl, S-alkyl, or N-alkyl;
O-alkenyl, S-alkenyl, or N-alkenyl;
O-alkynyl, S-alkynyl, or N-alkynyl;
O-alkyl-O-alkyl, 2'-F, 2'-OCH₃, or 2'-O(CH₂)₂OCH₃, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁-C₁₀, alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_n-NH₂, or -O(CH₂)_nON[(CH₂)_nCH₃]₂, wherein n and m are from 1 to about 10;
a modification at the 5' position comprising:
5'-vinyl, or 5'-methyl (R or S); or
a modification at the 4' position, 4'-S, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide; or
any combination thereof.

45. A cell comprising at least one unnatural DNA molecule comprising at least four unnatural base pairs, wherein the at least one unnatural DNA molecule encodes (i) a messenger ribonucleic acid (mRNA) molecule encoding an unnatural polypeptide and comprising at least first and second unnatural codons; and (ii) at least first and second transfer RNA (tRNA) molecules, the first tRNA molecule comprising a first unnatural anticodon and the second tRNA molecule comprising a second unnatural anticodon, wherein the at least four unnatural base pairs in the at least one DNA molecule are in sequence contexts such that the first and second unnatural codons of the mRNA molecule are complementary to the first and second unnatural anticodons, respectively.
46. The cell of claim 45, further comprising the mRNA molecule and the at least first and second tRNA molecules.
47. The cell of claim 46, wherein the at least first and second tRNA molecules are covalently linked to unnatural amino acids.
48. The cell of claim 47, further comprising the unnatural polypeptide.
49. A cell comprising:
- at least two different unnatural codon-anticodon pairs, wherein each unnatural codon-anticodon pair comprises an unnatural codon from an unnatural messenger RNA (mRNA) and an unnatural anticodon from an unnatural transfer ribonucleic acid (tRNA), said unnatural codon comprising a first unnatural nucleotide and said unnatural anticodon comprising a second unnatural nucleotide; and
 - at least two different unnatural amino acids each covalently linked to a corresponding unnatural tRNA.
50. The cell of claim 49, further comprising at least one unnatural DNA molecule comprising at least four unnatural base pairs (UBPs).
51. The cell of any one of claims 45-50, wherein the first unnatural nucleotide is positioned at a second or a third position of the unnatural codon.
52. The cell of claim 51, wherein the first unnatural nucleotide is complementarily base paired with the second unnatural nucleotide of the unnatural anticodon.

53. The cell of any one of claims 45-52, wherein the first unnatural nucleotide and the second unnatural nucleotide comprise first and second bases, respectively, independently selected from the group consisting of



54. The cell of any one of claims 45 or 47-53, wherein the at least four unnatural base pairs are independently selected from the group consisting of dCNMO/dTPT3, dNaM/dTPT3, dCNMO/dTAT1, or dNaM/dTAT1.
55. The cell of any one of claims 45 or 47-54, wherein the at least one unnatural DNA molecule comprises at least one plasmid.
56. The cell of any one of claims 45 or 47-54, wherein the at least one unnatural DNA molecule is integrated into genome of the cell.
57. The cell of any one of claims 47-56, wherein the at least one unnatural DNA molecule encodes an unnatural polypeptide.
58. The cell of any one of claims 45-57, wherein the cell expresses a nucleoside triphosphate transporter.

59. The cell of claim 58, wherein the nucleoside triphosphate transporter comprises the amino acid sequence of *PtNTT2*.
60. The method of claim 59, wherein the nucleoside triphosphate transporter comprises a truncated amino acid sequence of *PtNTT2*, optionally wherein the truncated amino acid sequence of *PtNTT2* is at least 80% identical to a *PtNTT2* encoded by SEQ ID NO.1.
61. The cell of any one of claims 45 to 60, wherein the cell expresses at least two tRNA synthetases.
62. The cell of claim 61, wherein the at least two tRNA synthetases are chimeric PylRS (chPylRS) and *M. jannaschii* AzFRS (*MjpAzFRS*).
63. The cell of any one of claims 45 to 62, wherein the cell comprises unnatural nucleotides comprising an unnatural sugar moiety.
64. The cell of claim 63, wherein the unnatural sugar moiety is selected from the group consisting of:
- a modification at the 2' position comprising
 - OH, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, or NH₂F;
 - O-alkyl, S-alkyl, or N-alkyl;
 - O-alkenyl, S-alkenyl, or N-alkenyl;
 - O-alkynyl, S-alkynyl, or N-alkynyl;
 - O-alkyl-O-alkyl, 2'-F, 2'-OCH₃, 2'-O(CH₂)₂OCH₃ wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁-C₁₀, alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_n-NH₂, or -O(CH₂)_nON[(CH₂)_nCH₃]₂, wherein n and m are from 1 to about 10;
 - a modification at the 5' position comprising:
 - 5'-vinyl, 5'-methyl (R or S); or
 - a modification at the 4' position, 4'-S, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group,

an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide; or
any combination thereof.

65. The cell of any one of claims 45 to 64, wherein at least one unnatural nucleotide base is recognized by an RNA polymerase during transcription.
66. The cell of any one of claims 45 to 65, wherein the cell translates at least one unnatural polypeptide comprising the at least two unnatural amino acids.
67. The cell of any one of claim 45 to 66, wherein the at least two unnatural amino acids are independently selected from the group consisting of N6-azidoethoxy-carbonyl-L-lysine (AzK), N6-propargylethoxy-carbonyl-L-lysine (PraK), N6-(propargyloxy)-carbonyl-L-lysine (PrK), p-azido-phenylalanine (*p*AzF), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, 2-amino-8-oxononanoic acid, 2-amino-8-oxooctanoic acid, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, 2-amino-8-oxononanoic acid, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, selenocysteine, N6-(((2-azidobenzyl)oxy)carbonyl)-L-lysine, N6-(((3-azidobenzyl)oxy)carbonyl)-L-lysine, and N6-(((4-azidobenzyl)oxy)carbonyl)-L-lysine.
68. The cell of any one of claims 45 to 67, wherein the cell is isolated.
69. The cell of any one of claims 45 to 68, wherein the cell is a prokaryote.
70. A cell line comprising the cell of any one of claims 45 to 69.