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Development of Strategies for the Site-Specific In Vivo Incorporation of Photoreactive Amino Acids: *p*-Azidophenylalanine, *p*-Acetylphenylalanine and Benzofuranylalanine

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Abstract—A major limitation of conventional site-directed mutagenesis is that substitutions are restricted to the 20 naturally occurring amino acids. While this problem can be circumvented in vitro to allow the site-specific incorporation of non-canonical amino acids, no similar in vivo methodologies yet exist. The main requirement for such a system is an aminoacyl-tRNA synthetase able to exclusively recognize a non-canonical amino acid and a suppressor tRNA. The engineering of such activities in aminoacyl-tRNA synthetases has proven to be problematic due to their high substrate specificity. Here we report progress towards the development of an antibody-based methodology to screen large mutant aminoacyl-tRNA synthetase libraries for specific recognition of the non-canonical photoreactive benzofuran amino acid [3-(5′-benzofuranyl)-alanine]. We also report the chemical synthesis and enantiomeric resolution of this amino acid.

Introduction

The use of site-directed mutagenesis to replace amino acids at any chosen position in a protein has greatly advanced our understanding of biological structure–function relationships in recent years. The only limitation of conventional site-directed mutagenesis is that substitutions are restricted to the 20 naturally occurring amino acids. It has been anticipated that expanding the range of available amino acids for translation will allow biochemists to 'tailor the structure of an amino acid to address a specific structure–function relation'.[1] This has long been possible through the use of amino acid analogs which mimic their natural counterparts during protein synthesis. However, this approach has had comparatively little impact on the field of protein engineering as it does not allow site-specific replacements to be made. While this problem has been overcome in vitro by using chemically and enzymatically aminoacylated tRNAs to incorporate numerous amino acid analogs, no comparable in vivo methodologies exist. Recent efforts to address this problem have focused on the engineering of aminoacyl-tRNA synthetases (AARSs) and tRNAs with a view to the development of a novel aminoacylation pathway (Fig. 1).

Engineering of aminoacylation pathways

Numerous quality control processes exist throughout translation which serve to minimize the number of errors during protein synthesis.[2] These processes share a common feature, namely that their activities are directed towards prevention of mistaking one natural substrate (either amino acid or tRNA) for another natural substrate. While this is to be expected, it means that the translational machinery can be subverted with unnatural substrates which are not normally encountered by the cell. For example, while elongation factor Tu (EF-Tu) can discriminate against a number of naturally occurring mischarged tRNAs, it nevertheless recognizes a vast range of synthetic aminoacyl-moieties which are subsequently incorporated into proteins (e.g. Ref. 3, reviewed in Ref. 4). In much the same way, while some synthetases display proofreading activity this may not

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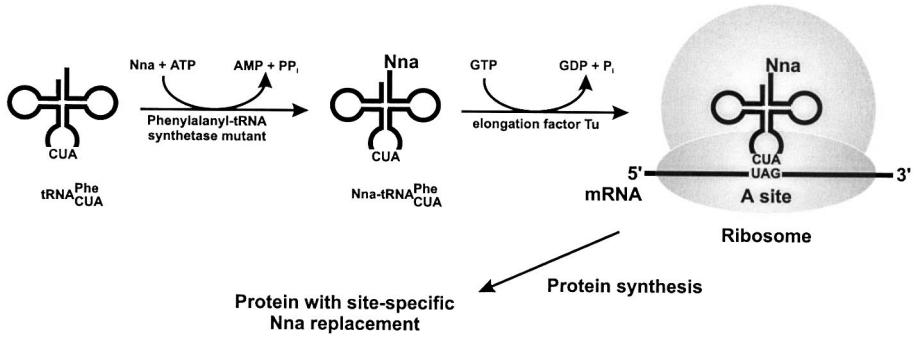


Figure 1. Hypothetical scheme for the in vivo site-specific incorporation of a non-canonical amino acid analog of phenylalanine (Nna). Following uptake by the cell, Nna is used as a substrate by a postulated phenylalanyl-tRNA synthetase mutant which recognizes both Nna and amber suppressor tRNA^{Phe} but no longer recognizes phenylalanine and wild-type tRNA^{Phe}.

be effective against non-canonical amino acids (e.g. Ref. 5). This flexibility in translation has led to a variety of experimental approaches aimed at broadening the range of amino acids available for translation, all of which require the synthesis of novel aminoacyl-tRNAs. The most successful of these methods has been the development of a technique which allows the site-specific incorporation of novel, non-canonical amino acids into proteins in vitro while overcoming restrictions of protein size associated with chemical peptide synthesis.[6] This method is based upon the translation of termination codons by aminoacylated suppressor tRNAs and the subsequent insertion of an amino acid at a stop site.[7] In this procedure a tRNA species containing the anticodon CUA is first purified, and then either chemically[1] or enzymatically aminoacylated[8] with the non-canonical amino acid of interest. The resulting aminoacyl-tRNA is then added to an in vitro translation reaction containing an mRNA where the codon UAG has been inserted at the desired site of non-canonical amino acid incorporation. The scope of this system has recently been extended to intact cells (*Xenopus* oocytes[9]) which should now allow its application to an even broader range of biological problems.

The more critical aspects of this system and its numerous applications have recently been extensively reviewed.[4,6,10,11] The major advantage of the system is that it allows the incorporation of a wide range of non-canonical amino acids at any codon amenable to mutagenesis to TAG. The overwhelming disadvantage is that it is strictly an in vitro technique and thus suffers limitations of both scale and scope of target proteins. Various examples have been reported of the in vivo translational incorporation of non-canonical amino acids into proteins.[12–16] Even though such non-canonical amino acid incorporation can be sustained in vivo, further modifications in the substrate specificity of aminoacyl-tRNA synthetases are required in order to achieve the site-specificity characteristic of the in vitro translation systems described above. Consequently, recent efforts to develop an in vivo system have focused on the engineering of aminoacyl-tRNA synthetases (AARSs) and tRNAs (e.g. Ref. 16) with a view to the development of a novel aminoacylation pathway which utilizes a non-canonical amino acid and a functionally redundant tRNA independently of the normal cellular translational machinery. Limited success has been achieved in solving the individual problems of exclusive recognition of amino acid and tRNA but no system yet exists which combines both of these features in the synthesis of a novel aminoacyl-tRNA. One potential route to overcome the problems inherent in modifying homologous components of translation is to introduce a heterologous AARS and tRNA.[14,17] The use of a heterologous aminoacylation pathway, however, may not completely overcome the potential problems of cross-reactivity due to the high degree of evolutionary conservation among both tRNAs and AARSs. For example, the inability of a phenyalanyl-tRNA synthetase (PheRS) mutant to recognize *p*-fluorophenyalanine has recently been exploited to direct incorporation of this amino acid analog at a programmed amber stop codon. This was achieved by the heterologous expression of a yeast suppressor tRNA^{Phe}/PheRS pair in an *Escherichia coli* strain resistant to *p*-fluorophenyalanine, allowing up to 75% incorporation of the non-canonical amino acid under certain growth conditions.[14] However, the utility of this system is limited due to the continued recognition of phenylalanine. Nevertheless, continuing developments in this area suggest that significant advances are to be expected in the near future. These include the continued development of the yeast GlnRS/tRNA^{Gln,16} and TyrRS/tRNA^{Tyr,17} systems in *E. coli*, where many of the inherent problems of tRNA substrate specificity have already been solved. It is also possible that application of the stringent mutagenesis and selection procedures developed with *E. coli* GlnRS and tRNA^{Gln,13}, combined with the extremely detailed information now available on the mechanisms underlying specificity in AARSs, could lead to considerable improvements in the site-specific incorporation of non-canonical amino acids in vivo.

Developing strategies for the site-directed incorporation of photoreactive amino acids

While many of the approaches to develop in vivo site-specific incorporation systems show much promise, it has become apparent that their ultimate success may be dependent on the development of improved screening techniques and the inclusion of functionally useful non-canonical amino acids. Consequently, our efforts have been focused on applying existing knowledge to the development of a system for the in vivo site-specific incorporation of a photoreactive non-canonical amino acid. Here we describe preliminary steps towards the selection of a suitable photoreactive amino acid and outline possible strategies for the

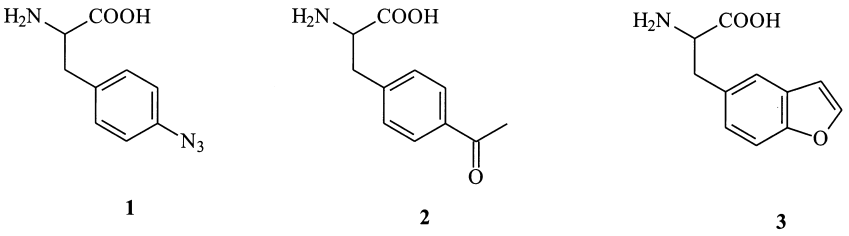


Figure 2. Photoreactive phenylalanine analogs used in this study. **1**, *p*-azidophenylalanine, **2**, *p*-acetylphenylalanine, **3**, benzofuranylalanine.

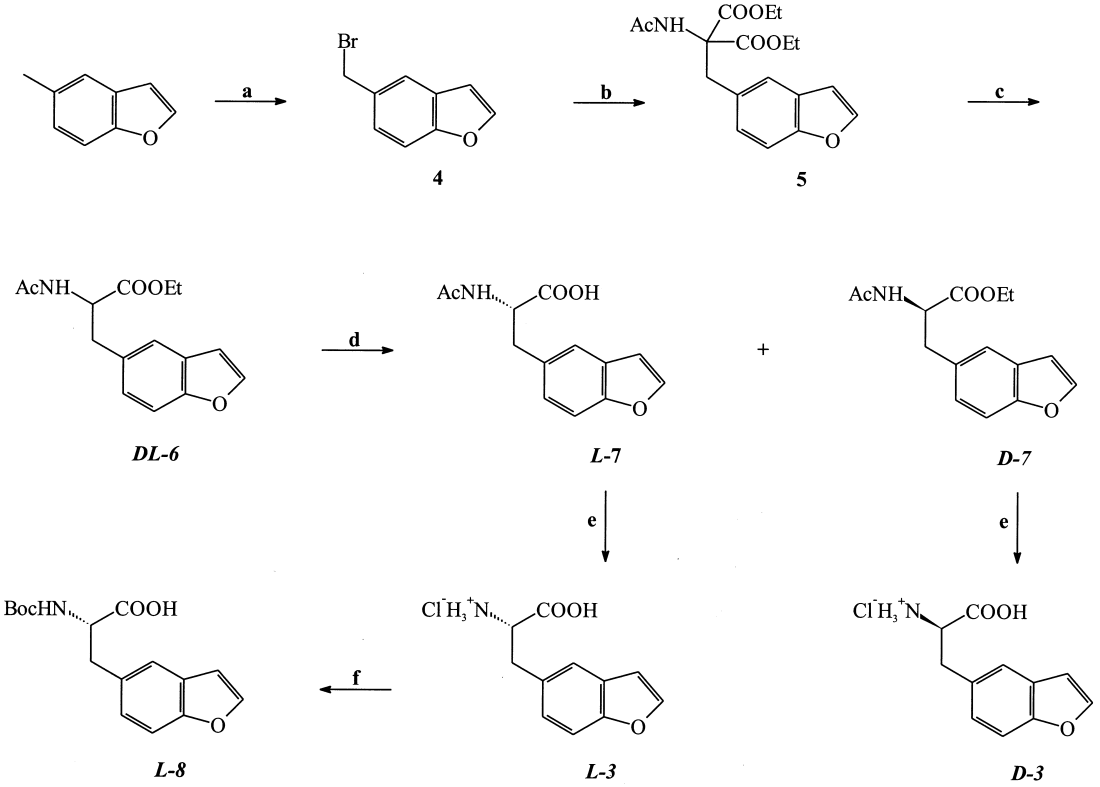
development of a site-specific incorporation system based upon such a non-canonical amino acid.

Results and Discussion

Selection of a candidate aminoacyl-tRNA synthetase

The possible utility of three photoreactive phenylalanine analogs as substrates was initially tested in vitro. The underlying rationale for this approach was based upon previous studies which showed that the active site of *E. coli* phenylalanyl-tRNA synthetase (PheRS) could be mutated to accommodate a variety of *para*-substituted phenylalanine analogs.[5] The alanine at position 294 (Ala294) in the α-subunit has been implicated as a determinant of amino acid specificity by determining the size of the substrate binding pocket.[5,18] Replacement of Ala294 by either glycine or serine, which would increase or decrease the size of the binding pocket, respectively, reduced the specificity for phenylalanine. The Gly294 mutant showed a relaxed specificity towards synthetic *para*-halogenated phenylalanine analogs although it still recognized phenylalanine. The size of the binding pocket apparently affects the substrate specificity since the apparent dissociation constant (K_M) increased in direct relation to the van der Waals radius of the *para*-group.[5] Therefore, Gly294 PheRS was of potential interest as a means of developing a system for in vivo site specific incorporation of non-canonical amino acids. The choice of Gly294 PheRS is further supported by its recent use for the in vivo production of *p*-bromophenylalanine substituted dihydrofolate reductase.[19]

The strategy employed was to initially select a photoreactive amino acid that would be accepted by Gly294 PheRS and to then use this mutant enzyme as the starting point for development of an in vivo site-specific incorporation system. As useful photoreactive analogs should absorb light at wavelengths above 300 nm, selection was necessarily limited to aromatic non-canonical amino acids, further emphasizing the utility of the PheRS system.



Scheme 1. Chemical synthesis of benzofuranylalanine and resolution of enantiomers. *Reagents and conditions* (see text for details): (a) NBS, Bz_2O_2, CCl_4, reflux, (b) NaOEt, diethyl acetamidomalonate, EtOH, 50°C, (c) LiCl, H_2O, DMF, 140°C, (d) Carlsberg subtilisin, 20% DMSO, PBS, room temperature, (e) 5N HCl, reflux, (f) NaOH, Boc_2O, H_2O, room temperature.

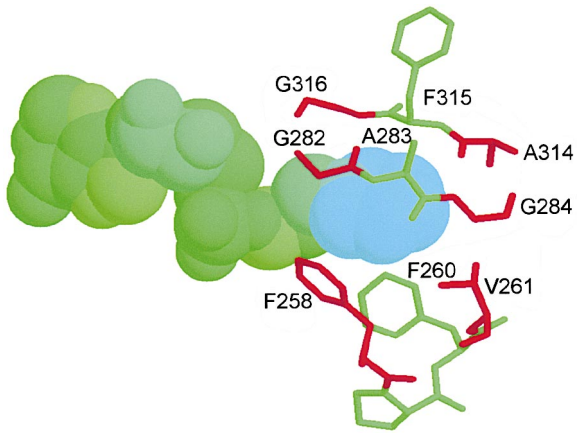


Figure 3. The interactions between the aromatic moiety of phenylalaninyl-adenylate and the active site of *T. thermophilus* PheRS.[20] Phenylalaninyl-adenylate is shown as a space-filling model, with the aromatic moiety in cyan. All the residues shown are involved in hydrophobic interactions with the phenyl-group. Drawn with Rasmol (Roger Sayle, Glaxo, Greenford, UK).

Selection of photoreactive non-canonical amino acids

An increase of the size of the substituted *para*-group in phenylalanine reduces the ability of Gly294 PheRS to accept amino acid analogs. This indicates that steric considerations determine the specificity of the synthetase, with *p*-bromophenylalanine the largest analog accepted by the mutant PheRS (for *p*-iodophenylalanine, no conclusive results were obtained due to poor solubility).[5] Thus, besides being photoreactive, the reactive group in the *para*-position should not exceed the van der Waals radius of *p*-bromophenylalanine. In addition, the non-canonical amino acid should be structurally close to phenylalanine and should have a sufficient photochemical activity at wavelengths above 300 nm where natural amino acids do not absorb. Based upon these considerations, three different photoreactive phenylalanine analogs, an azido, an acetophenone and a benzofuran were synthesized and selected for further investigation (Fig. 2). The synthesis of benzofuranylalanine (Bfa) is detailed below (experimental) and is outlined in Scheme 1. Enantiomeric resolution was accomplished enzymatically using Carlsberg subtilisin.

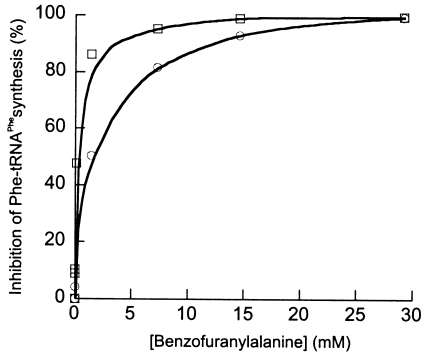


Figure 4. Inhibition of L-[^{3}H] Phe-tRNA^{Phe} synthesis by L-Bfa and D-Bfa. Aminoacylation was performed with Gly294 PheRS in the presence of increasing concentrations of either L-Bfa (□) or D-Bfa (○). Data shown represent percent inhibition of aminoacyl-tRNA synthesis, 100% defined as the level of product synthesis in the absence of Bfa.

In vitro aminoacylation of non-canonical amino acids by PheRS

Wild-type and Gly294 PheRS were partially purified, and used for in vitro acylation in the presence of non-canonical phenylalanine amino acid analogs as competitors. Unlabelled -DL-*p*-azidophenylalanine, -DL-*p*-acetylphenylalanine, and -DL-Bfa were tested for their ability to compete with acylation of *E. coli* tRNA^{Phe} by radiolabelled phenylalanine.

50% Inhibition of Phe-tRNA^{Phe} synthesis was observed at 3 mM-DL-*p*-azidophenylalanine for Gly294 PheRS and 180 mM for wild-type. Similarly, -DL-Bfa showed 50% inhibition of Phe-tRNA^{Phe} by Gly294 PheRS at a concentration of 300 μM (50% inhibition of wild-type was seen at 180 mM). The high selectivity of *p*-azidophenylalanine and Bfa for inhibition of aminoacylation by Gly294 PheRS was not seen for *p*-acetylphenylalanine. When taken together with previous results, these data suggest that the active site of Gly294 PheRS is able to accommodate Bfa or *p*-azidophenylalanine, but not *p*-acetylphenylalanine, perhaps as a result of its larger amino acid binding pocket compared to wild-type PheRS. This possibility was further supported by examination of the co-crystal structure of *Thermus thermophilus* PheRS complexed phenylalaninyl-adenylate,[20] which illustrates how larger *para*-groups can be accommodated at the active site (Fig. 3).

While inhibition of steady-state aminoacylation and molecular modeling suggest that both Bfa and *p*-azidophenylalanine can be accommodated in the active site of Gly294 PheRS, these data do not indicate whether either non-canonical amino acid is a substrate for aminoacylation of tRNA^{Phe}. This was further investigated for Bfa by probing the stereospecificity of the inhibition of Phe-tRNA^{Phe} synthesis. The D- and L-Bfa enantiomers were enzymatically resolved and used separately for in vitro competition assays. No difference in competition of aminoacylation between D- and L-Bfa was observed for the wild type enzyme (data not shown), whereas Gly294 PheRS was more strongly inhibited by L-Bfa (Fig. 4). The stereo-specificity of the inhibition of aminoacylation is consistent with the high degree of selectivity of *E. coli* PheRS for L-phenylalanine compared to D-phenylalanine,[21] suggesting that L-Bfa is a substrate for aminoacylation.

Physiological effects of benzofuranylalanine

The utility of non-canonical amino acids for the development of site-specific in vivo incorporation systems is dependent on the cellular uptake of such compounds. The possible uptake by *E. coli* of Bfa was studied by measuring its effect on cell growth. These studies were performed in parallel with isogenic *E. coli* strains which specifically produce either wild-type (KA4) or Gly294 PheRS (KA3). -DL-Bfa inhibited growth of *E. coli* KA3 at concentrations above 1.5 mM, but had no effect on *E. coli* KA4 even at a concentration of 45 mM. Testing of the purified D-and L- enantiomers of Bfa showed that inhibition of growth of *E. coli* KA3 was exclusively observed with L-Bfa (at concentrations of 1.5 mM and above). This is in good agreement with the in

vitro stereospecific inhibition of Phe-tRNA^{Phe} synthesis by L-Bfa (see above).

Development of a system to site-specifically incorporate benzofuranylalanine in vivo

Preliminary data indicate that L-Bfa is a substrate for *E. coli* PheRS in vivo. Before proceeding further, it is necessary to confirm that these properties lead to the co-translational insertion of Bfa in vivo in a suitable expression system. While this can be accomplished in a number of ways, the recent description of a Gly294 PheRS-based system for the in vivo incorporation of *para*-substituted phenylalanine analogs is an obvious choice.[19] The use of such a system should allow the ready synthesis and biophysical analysis of a Bfa-containing target protein.

The more pressing question concerns how an in vivo system can be developed which will allow the site-specific incorporation of Bfa at any chosen position in a protein. Based upon existing in vitro systems, the most obvious route is to attempt to direct insertion at an engineered amber stop codon. As detailed above this would require modification of PheRS such that it efficiently recognizes Bfa and tRNA$^{Phe}_{CUA}$, but no longer recognizes phenylalanine and tRNA^{Phe}. Previous studies have shown that the issue of tRNA recognition can be readily resolved by the use of a heterologous system (e.g. the co-expression of yeast PheRS and yeast tRNA^{Phe} in *E. coli*).[14]

The engineering of PheRS to exclusively recognize Bfa is more problematic. Efforts to systematically change the amino acid specificity of other aminoacyl-tRNA synthetases based upon crystal structures of enzyme–substrate complexes have only met with partial success. For example, while improvements in non-cognate amino acid recognition have been reported for glutaminyl-tRNA synthetase (GlnRS),[22,23] tryptophanyl-tRNA synthetase (Prætorius-Ibba, unpublished results) and tyrosyl-tRNA synthetase,[24] in all cases the mutant proteins retained a preference for their cognate substrates. This suggests that it may be necessary to adopt a combinatorial library construction and screening strategy, perhaps such as that applied to GlnRS.[13] In such an approach, Gly294 PheRS would be used as a starting point and libraries constructed by randomization of other residues implicated in determining the specificity of phenylalanine binding (Fig. 3). Two possible strategies could be employed to screen such libraries for functionality, rescue of a suppressor-dependent phenotype[16] or direct screening for specific recognition of Bfa. Since initial efforts with suppressor-based systems have proved problematic (J.N. Nielsen and P.E. Nielsen, unpublished results), we are currently attempting to develop an antibody-based positive screen for Bfa utilization. Such a screen will utilize a monoclonal antibody raised against L-Bfa chemically incorporated into a hexamer peptide, which is currently under construction. Studies with other photoreactive non-canonical amino acids (J.N. Nielsen, C. Behrens and P.E. Nielsen, unpublished results) have suggested that such an antibody should provide sufficient sensitivity and specificity for the development of a library screening strategy.

Future Prospects

Recent years have seen an increasing interest in the development of experimental strategies which would allow the in vivo site-specific incorporation of non-canonical amino acids. To date, the main obstacle to the successful development of these new methods has been engineering of the necessary amino acid substrate specificity into aminoacyl-tRNA synthetases. However, the integration of a rational substrate selection strategy with recent advances in combinatorial protein mutagenesis and antibody-based positive screening suggest that it may eventually be possible to derive novel aminoacyl-tRNA synthetases with suitable activities.

Experimental

General

The overexpression of *E. coli* wild-type and Gly294 PheRS have been described.[5] *E. coli* tRNA^{Phe} was purchased from Sigma (St. Louis, MO). Media for bacterial growth and molecular biology protocols were standard unless otherwise noted.[25] All reagents (Aldrich or Fluka) and solvents (LabScan) were of standard quality and used without further purification unless indicated. NMR spectra were recorded on either a Bruker 250 MHz or a Varian 400 MHz spectrometer with chemical shifts in ppm relative to tetramethylsilane (^{1}H and ^{13}C NMR) as internal standard. Melting points were recorded on a Büchi melting point apparatus and are uncorrected. Flash chromatography was performed using Silica gel 60 (0.040–0.063 mm). Microanalysis were performed at The Microanalytical Laboratory, Department of Chemistry, University of Copenhagen.

Synthesis of non-canonical amino acids and resolution of enantiomers

p-Azidophenylalanine and *p*-acetylphenylalanine were synthesized as previously described.[26–28] The synthesis of benzofuranylalanine is summarized in Scheme 1, and the individual steps as annotated are detailed below.

5-(Bromomethyl)benzofuran (4). 5-Methylbenzofuran[29] (1.00 g, 8.0 mmol) was dissolved in carbon tetrachloride (50 ml). Benzoyl peroxide (50 mg) and *N*-bromosuccinimide (1.42 g, 8.0 mmol) were added and the mixture was heated under reflux for 48 h. The solvent was then removed by rotary evaporation in vacuo, and the residual oil purified by flash chromatography (petroleum ether). Fractions containing one spot on TLC (R_f=0.35 in *n*-hexane) were pooled and the solvent removed in vacuo to give the title product as a crystalline solid. Yield: 620 mg (38%); ^{1}H NMR ($CDCl_3$) δ 4.64 (s, 2H), 6.75 (dd, 1H, J=1 Hz, J=2 Hz), 7.34 (dd, 1H, J=2 Hz, J=9 Hz), 7.48 (d, 1H, J=9 Hz), 7.64 (d, 1H, J=2 Hz), 7.67 (d, 1H, J=2 Hz); ^{13}C NMR ($CDCl_3$) δ 34.16, 106.45, 111.66, 121.72, 125.47, 127.67, 132.37, 145.75, 154.60.

2-Acetamino-2-carbethoxy-3-(benzofuran-5-yl)propanoic acid ethyl ester (5). To a solution of sodium ethoxide, prepared by dissolving sodium (131 mg, 5.68 mmol) in

absolute ethanol (25 ml) with heating, was added diethyl acetamidomalonate (1.85 g, 8.52 mmol), and the mixture was stirred for 5 min at room temperature. A solution of 5-bromomethylbenzofuran (**4**) (600 mg, 2.85 mmol) in absolute ethanol (2 ml) was then added and the mixture was heated to 50°C on a water bath. After 20 min the solution was cooled and taken to dryness by rotary evaporation in vacuo. The residue was dissolved in ethanol (5 ml) and water (50 ml) was added. The precipitate was collected and recrystallized from ethanol/water. Yield: 904 mg (90%); mp 101–102°C; ^{1}H NMR (DMSO-d_6) δ 1.18 (t, 6H, J=7 Hz), 1.96 (s, 3H), 3.53 (s, 2H), 4.16 (q, 4H, J=7 Hz), 6.90 (s, 1H), 6.92 (d, 1H, J=8 Hz), 7.25 (bs, 1H), 7.49 (d, 1H, J=8 Hz), 7.94 (s, 1H), 8.05 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 13.92, 22.28, 37.53, 61.83, 67.33, 106.70, 110.88, 122.47, 126.27, 127.38, 129.71, 146.22, 153.66, 167.16, 169.52; MS (FAB+) 348.17 (MH^+) (calcd for $C_{18}H_{21}NO_6$: 347.1369). Anal. calcd for $C_{18}H_{21}NO_6$: C, 62.24; H, 6.09; N, 4.03. Found: C, 62.19; H, 6.11; N, 3.97.

***N*-Acetyl-3-(5′-benzofuranyl)-DL-alanine ethyl ester (DL-6).** 2-Acetamino-2-carbethoxy-3-(benzofuran-5-yl)-propanoic acid ethyl ester (**5**) (4.95 g, 14.3 mmol) and lithium chloride (2.53 g, 57.3 mmol) were dissolved in a mixture of water (2.2 ml) and DMF (120 ml) and heated to 140°C for 16 h. The solvent was then removed in vacuo, and the solid residue taken up in water (50 ml). The aqueous phase was extracted with ethyl acetate (3×100 ml), and the combined organic layers dried over sodium sulfate. The solvent was removed by rotary evaporation in vacuo, and the residual oil purified by flash chromatography (step gradient of CH_2Cl_2 followed by 10% MeOH/CH_2Cl_2). Fractions containing one spot on TLC (R_f=0.4 in 5% MeOH/CH_2Cl_2) were pooled and taken to dryness. The solid obtained was subsequently recrystallised from ethanol/water. Yield: 3.114 g (79%); ^{1}H NMR ($CDCl_3$) δ 1.21 (t, 3H, J=7 Hz), 1.97 (s, 3H), 3.19 (dd, 2H, J=6 Hz, J=3 Hz), 4.15 (q, 2H, J=7 Hz), 4.86 (q, 1H, J=6 Hz), 6.09 (bs, 1H), 6.69 (s, 1H), 7.01 (d, 1H; J=8 Hz), 7.31 (s, 1H), 7.39 (d, 1H, J=8 Hz), 7.59 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.99, 22.96, 37.64, 53.41, 61.34, 106.22, 111.12, 121.53, 125.35, 127.52, 130.16, 145.21, 153.98, 169.55, 171.50; MS (FAB+) 276.130 (MH^+) (calcd for $C_{15}H_{17}NO_4$: 275.1157). Anal. calcd for $C_{15}H_{17}NO_4$: C, 65.44; H, 6.22; N, 5.08. Found: C, 65.28; H, 6.18; N, 4.95.

***N*-Acetyl-3-(5′-benzofuranyl)-L-alanine (L-7).** *N*-Acetyl-3-(5′-benzofuranyl)-DL-alanine (**DL-6**) (2.40 g, 8.7 mmol) was dissolved in a mixture of DMSO (25 ml) and 100 ml of 0.1 M phosphate buffer solution (100 mM KH_2PO_4, NaOH, pH=7.5). Carlsberg subtilisin (20 mg dry weight) was added and the mixture was stirred overnight at room temperature. HPLC analysis of a small aliquot indicated a 53% conversion to the free acid form (L-form). Non reacted ethyl ester (D-form) was removed by extraction with ethyl acetate (4×100 ml). The solvent was removed by rotary evaporation in vacuo, the residue obtained was dissolved in water (40 ml) and the pH was adjusted to 2.5 by addition of diluted hydrochloric acid. The aqueous phase was extracted with ethyl acetate (4×100 ml), and the organic layer washed with brine and dried over anhydrous sodium sulfate. Evaporation to dryness in vacuo afforded the title product as a yellow oil which slowly crystallized upon prolonged standing. A sample suitable for elemental analysis was obtained by performing one additional crystallization from water/ethanol. Yield: 884 mg (41%); mp 147–150°C; $[\alpha]_D^{20}$=42.5 c=1, H_2O); ^{1}H NMR (DMSO-d_6) δ 1.77 (s, 3H), 2.53 (d, 1H, J=1.0 Hz), 2.92 (dd, 1H, J=9.0 Hz, J=13.5 Hz), 3.12 (dd, 1H, J=5.0 Hz, J=13.5 Hz), 4.43 (ddd, 1H, J=1.0 Hz, J=5.0 Hz, J=9.0 Hz), 6.90 (d, 1H, J=1.0 Hz), 7.16 (dd, 1H, J=2.5 Hz, J=8.5 Hz), 7.46 (d, 1H, J=2.5 Hz), 7.48 (d, 1H, J=3 Hz), 7.95 (d, 1H, J=2.5 Hz), 8.17 (d, 1H, J=8.5 Hz); ^{13}C NMR (DMSO-d_6) δ 22.42, 36.81, 54.04, 106.64, 110.86, 121.49, 125.58, 127.22, 132.25, 146.11, 153.30, 169.25, 173.19; MS (FAB+) 248.071 (MH^+) (calcd for $C_{13}H_{13}NO_4$: 247.084). Anal. calcd for $C_{13}H_{13}NO_4.(H_2O)_{0.5}$ C, 60.93; H, 5.50; N, 5.46. Found: C, 60.93; H, 5.30; N, 5.33.

3-(5′-Benzofuranyl)-D-alanine hydrochloride (D-3). *N*-Acetyl-3-(5′-benzofuranyl)-D-alanine ethyl ester (**D-7**) (1.02 g, 3.7 mmol) was dissolved in 5N HCl (50 ml) and heated under reflux for 4 h. After cooling, the solvent was removed in vacuo and the crystalline residue dissolved in absolute ethanol (10 ml). Upon addition of dry diethyl ether (50 ml) the hydrochloride salt of the title product precipitated out of solution. The salt was filtered off and washed several times with dry diethyl ether. Yield: 438 mg (49%); mp 236–238°C; $[\alpha]_D^{20}$=+15.3 c=1, H_2O), e.e.=97.3% determined by derivatization with L-N^{α}-(2,4-dinitro-5-fluorophenyl)alaninamide (vide infra); ^{1}H NMR (D_2O) δ 3.22 (dd, 1H, J=8.0 Hz, J=14.5 Hz), 3.35 (dd, 1H, J=6.5 Hz, J=14.5 Hz), 4.27 (dd, 1H, J=6.5 Hz, J=8.0 Hz), 6.80 (d, 1H, 1.0 Hz), 7.16 (d, 1H, J=8.0 Hz), 7.47 (d, 1H, J=8.0 Hz), 7.49 (s, 1H), 7.70 (d, 1H, J=1.0 Hz); ^{13}C NMR (D_2O) δ 35.43, 54.45, 106.40, 111.73, 122.04, 125.30, 127.92, 128.41, 146.28, 154.03, 171.49.

3-(5′-Benzofuranyl)-L-alanine hydrochloride (L-3). *N*-Acetyl-3-(5′-benzofuranyl)-L-alanine (**L-7**) (700 mg, 2.83 mmol) was dissolved in 5N HCl (50 ml) and heated under reflux for 4 h. After cooling, the solvent was removed in vacuo and the crystalline residue dissolved in absolute ethanol (10 ml). Upon addition of dry diethyl ether (50 ml) the hydrochloride salt of the title product precipitated out of solution. The salt was filtered off and washed several times with dry diethyl ether. Yield: 606 mg (88%); mp 234–236°C, $[\alpha]_D^{20}$=−12.9 c=1, H_2O), e.e.=93.3% determined by derivitization with L-N^{α}-(2,4-dinitro-5-fluorophenyl)-alaninamide (vide infra); ^{1}H NMR (D_2O) δ 3.22 (dd, 1H, J=8.0 Hz, J=14.5 Hz), 3.35 (dd, 1H, J=6.5 Hz, J= 14.5 Hz), 4.27 (dd, 1H, J=6.5 Hz, J=8.0 Hz), 6.80 (d, 1H, 1.0 Hz), 7.16 (d, 1H, J=8.0 Hz), 7.47 (d, 1H, J=8.0 Hz), 7.49 (s, 1H), 7.70 (d, 1H, J=1.0 Hz); ^{13}C NMR (D_2O) δ 35.43, 54.45, 106.40, 111.73, 122.04, 125.30, 127.92, 128.41, 146.28, 154.03, 171.49; MS (FAB+) 206.100 (MH^+) (calcd for $C_{11}H_{11}NO_3$: 205.074).

***N-tert*-Butyloxycarbonyl-3-(5′-benzofuranyl)-L-alanine (L-8).** 3-(5′-Benzofuranyl)-L-alanine hydrochloride (**L-3**) (350 mg, 1.45 mmol) was dissolved in water (20 ml). Addition of 1N NaOH (2.90 ml, 2.90 mmol) was followed by di-*tert*-butyl dicarbonate (349 mg, 1.60 mmol). The mixture was stirred at room temperature for 24 h, then

extracted with ethyl acetate (2×20 ml). The pH of the aqueous phase was adjusted to 3.0 by addition of 2N hydrochloric acid and extracted with ethyl acetate (3×50 ml). The combined organic layers were dried with anhydrous sodium sulfate, filtered and evaporated in vacuo to dryness. The residual oil thus obtained was further purified by flash chromatography (10% MeOH/CH_2Cl_2). Fractions containing one spot on TLC R_f=0.3 in 10% MeOH/CH_2Cl_2) were pooled and the solvent removed in vacuo to give the title product as a clear oil. The oil was dissolved in water and lyophilized to give a white crystalline solid. Yield: 260 mg (59%); mp 128–130°C; ^{1}H NMR (DMSO-d_6) δ 1.30 (s, 9H), 2.98 (dd, 1H, J=7.5 Hz, J=13.0 Hz), 3.18 (dd, 1H, J=5.0 Hz, J=13.0 Hz), 3.97 (m, 1H), 6.16 (d, 1H, J=7.0 Hz), 6.83 (d, 1H, J=2.0 Hz), 7.12 (dd, 1H, J=2.0 Hz, J=10.5 Hz), 7.40 (d, 1H, J=10.5 Hz), 7.41 (s, 1H), 7.88 (d, 1H, J=2.0 Hz); ^{13}C NMR (DMSO-d_6) δ 27.04, 36.19, 55.31, 76.26, 105.31, 109.13, 118.41, 120.51, 124.80, 125.66, 132.29, 144.50, 151.89, 153.56; MS (FAB+) 306.98 (MH^+) (calcd for $C_{16}H_{19}NO_5$: 305.13).

Standard protocol for determination of e.e.

To a solution of the amino acid hydrochloride derivative (5 μmol) in water (60 μl), was added a 1% solution of L-N^{α}-(2,4-dinitro-5-fluorophenyl)alaninamide in acetone (100 μl) followed by 1 M $NaHCO_3$ (80 μl). The mixture was stirred for one hour at 40°C, then neutralized with 2N HCl (40 μl). After dilution with DMF (1000 μl) samples were analyzed by RP-HPLC (5 μl injection volume).

Aminoacylation assays

Aminoacylation assays were performed as described.[5] The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 2 mM reduced glutathione, 2 mM ATP, 30 μM ^{3}H-Phe and 0.2 μM $tRNA^{Phe}$. After addition of enzyme, reactions were incubated at 28°C and samples periodically removed and processed as described.[5] All assays were performed in duplicate, and the observed values vary from the mean values by less than 10%.

Growth competition assays

Initial screening of the effect of non-canonical amino acids on growth of *E. coli* strains was performed by inoculation into M9 minimal medium containing various concentrations of the particular non-canonical amino acid followed by growth overnight at 37°C. The effects of L- and D-Bfa were further investigated by inoculation (10% v/v) of overnight cultures into M9 medium containing various concentrations of the non-canonical amino acid. These cultures were then incubated at 37°C and growth monitored.

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References

1. Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182–188.
2. Ibba, M.; Söll, D. *Science* **1999**, *286*, 1893–1897.
3. Hohsaka, T.; Sato, K.; Sisido, M.; Takai, K.; Yokoyama, S. *FEBS Lett.* **1993**, *335*, 47–50.
4. Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 435–462.
5. Ibba, M.; Kast, P.; Hennecke, H. *Biochemistry* **1994**, *33*, 7107–7112.
6. Cornish, V. W.; Schultz, P. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 601–607.
7. Murgola, E. J. *tRNA: Structure, Biosynthesis, and Function*; Söll, D., RajBhandary, U. L., Eds.; ASM Press: Washington, DC, 1995, pp 491–510.
8. Sonar, S.; Lee, C. P.; Coleman, M.; Patel, N.; Liu, X.; Marti, T.; Khorana, H. G.; RajBhandary, U. L.; Rothschild, K. J. *Nat. Struct. Biol.* **1994**, *1*, 512–517.
9. Nowack, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W. G.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A. *Science* **1995**, *268*, 439–442.
10. Benner, S. A. *Trends Biotechnol.* **1994**, *12*, 158–163.
11. Ibba, M. *Biotech. Genet. Rev.* **1996**, *13*, 197–216.
12. Ibba, M.; Hennecke, H. *FEBS Lett.* **1995**, *364*, 272–275.
13. Liu, D. R.; Magliery, T. J.; Pastrnak, M.; Schultz, P. G. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10092–10097.
14. Furter, R. *Protein Sci.* **1998**, *7*, 419–426.
15. Budisa, N.; Minks, C.; Alefelder, S.; Wenger, W.; Dong, F.; Moroder, L.; Huber, R. *FASEB J.* **1999**, *13*, 41–51.
16. Liu, D. R.; Schultz, P. G. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4780–4785.
17. Ohno, S.; Yokogawa, T.; Fujii, I.; Asahara, H.; Inokuchi, H.; Nishikawa, K. *J. Biochem.* **1998**, *124*, 1065–1068.
18. Kast, P.; Hennecke, H. *J. Mol. Biol.* **1991**, *222*, 99–124.
19. Sharma, N.; Furter, R.; Kast, P.; Tirrell, P. *FEBS Lett.* **2000**, *467*, 37–40.
20. Reshetnikova, L.; Moor, N.; Lavrik, O.; Vassylyev, D. G. *J. Mol. Biol.* **1999**, *287*, 555–568.
21. Santi, D. V.; Webster Jr., R. W. *J. Med. Chem.* **1976**, *19*, 1276–1279.
22. Agou, F.; Quevillon, S.; Kerjan, P.; Mirande, M. *Biochemistry* **1998**, *37*, 11309–11314.
23. Hong, K. W.; Ibba, M.; Soll, D. *FEBS Lett.* **1998**, *434*, 149–154.
24. de Prat Gay, G.; Duckworth, H. W.; Fersht, A. R. *FEBS Lett.* **1993**, *318*, 167–171.
25. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1990.
26. DeGraw, J. I.; Cory, M.; Skinner, W. A.; Theisen, M. C.; Mitoma, C. *J. Med. Chem.* **1968**, *11*, 225–227.
27. Schwyzer, R.; Caviezel, M. *Helv. Chim. Acta* **1971**, *54*, 1395–1400.
28. Wieland, T.; von Dungen, A.; Birr, C. *Liebigs Ann. Chem.* **1971**, *752*, 109–114.
29. Barker, P.; Finke, P.; Thompson, K. *Synth. Commun.* **1989**, *19*, 257–265.