3-5-2004

Photoreactive Bicyclic Amino Acids as Substrates for Mutant Escherichia coli Phenylalanyl-tRNA Synthetases

Thomas Bentin
University of Copenhagen

Ramin Hamzavi
University of Copenhagen

Jahan Salomonsson
University of Copenhagen

Hervé Roy
The Ohio State University

Michael Ibba
Chapman University, ibba@chapman.edu

See next page for additional authors
Follow this and additional works at: https://digitalcommons.chapman.edu/sees_articles

Part of the Amino Acids, Peptides, and Proteins Commons, Biochemistry Commons, Cellular and Molecular Physiology Commons, Molecular Biology Commons, Nucleic Acids, Nucleotides, and Nucleosides Commons, and the Other Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation
Bentin, T., Hamzavi, R., Salomonsson, J., Roy, H., Ibba, M. and Nielsen, P.E. (2004) Photoreactive bicyclic amino acids as substrates for mutant Escherichia coli phenylalanyl-tRNA synthetases. J. Biol. Chem. 279, 19839-19845. https://doi.org/10.1074/jbc.M401278200

This Article is brought to you for free and open access by the Science and Technology Faculty Articles and Research at Chapman University Digital Commons. It has been accepted for inclusion in Biology, Chemistry, and Environmental Sciences Faculty Articles and Research by an authorized administrator of Chapman University Digital Commons. For more information, please contact laughtin@chapman.edu.
Photoreactive Bicyclic Amino Acids as Substrates for Mutant *Escherichia coli* Phenylalanyl-tRNA Synthetases

Comments
This article was originally published in *Journal of Biological Chemistry*, volume 279, in 2004. 
https://doi.org/10.1074/jbc.M401278200

Copyright
American Society for Biochemistry and Molecular Biology

Authors
Thomas Bentin, Ramin Hamzavi, Jahan Salomonsson, Hervé Roy, Michael Ibba, and Peter E. Nielsen
Photoreactive Bicyclic Amino Acids as Substrates for Mutant Escherichia coli Phenylalanyl-tRNA Synthetases*

Thomas Bentin‡, Ramin Hamzavi‡, Johan Salomonsson‡, Hervé Roy§, Michael Ibba§, and Peter E. Nielsen‡‡

From the ‡Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Blegdammsvej 3c, 2200 Copenhagen N, Denmark and §Department of Microbiology, The Ohio State University, Columbus, Ohio 43210-1292

Unnatural amino acids carrying reactive groups that can be selectively activated under non-invasive biologically benign conditions are of interest in protein engineering as biological tools for the analysis of protein-protein and protein-nucleic acids interactions. The double ring system phenylalanine analogues benzofuranylanine and benzotriazolylalanine were synthesized, and their photolability was investigated by UV irradiation at 254, 320, and 365 nm. Although both showed photo reactivity, benzofuranylanine appeared as the most promising compound because this amino acid was activated by UVA (long wavelength) irradiation. These amino acids were also tested for in vitro charging of tRNA\(^{\text{phe}}\) and for protein mutagenesis via the phenylalanyl-tRNA synthetase variant \(\alpha\)A294G that is able to facilitate in vivo protein synthesis using a range of \(\text{para}\)-substituted phenylalanine analogues. The results demonstrate that benzofuranylanine, but not benzotriazolylalanine, is a substrate for phenylalanine tRNA synthetase \(\alpha\)A294G, and matrix-assisted laser desorption ionization time-of-flight analysis showed it to be incorporated into a model protein with high efficiency. The in vivo incorporation into a target protein of a bicyclic phenylalanine analogue, as described here, demonstrates the applicability of phenylalanine tRNA synthetase variants in expanding the scope of protein engineering.

The use of unnatural amino acids for protein engineering is a rapidly developing technology that adds new dimensions to conventional mutagenesis by allowing introduction of novel chemical and biological functionality into proteins (1). *Escherichia coli* has been the organism most widely used for in vivo unnatural amino acid incorporation (e.g., 2–5), but eukaryotic host systems (6–8) have also recently been described. The development of all such systems is dependent on engineering the substrate specificity of the aminoacyl-tRNA synthetase to recognize amino acid analogues that are close structural mimics of the cognate amino acid (2, 3). For instance, an \(\alpha\) e.coli phenylalanine tRNA synthetase (PheRS\(^{\text{e.coli}}\)) variant carrying a single Ala \(\rightarrow\) Gly amino acid substitution at \(\alpha\)-subunit residue 294 (PheRS-\(\alpha\)A294G) (10) displays relaxed substrate specificity in vivo toward a number of \(\text{para}\)-substituted phenylalanine mimics. Examples include several halogenated phenylalanines including \(p\)-chlorophenylalanine (2), \(p\)-bromophenylalanine (11) and \(p\)-iodophenylalanine as well as \(p\)-cyano-phenylalanine, \(p\)-ethynlyphenylalanine, \(p\)-azidophenylalanine, and 2-, 3-, and 4-pyridylalanine (12). Introducing the additional mutation Thr \(\rightarrow\) Gly in position 251 (PheRS-\(\alpha\)T251G/A294G) further enlarges the amino acid binding pocket, which provides space for phenylalanine analogues carrying modifications on the benzene ring and allows activation of still larger unnatural amino acids such as \(p\)-acetylphenylalanine (3).

Based upon previous studies of proteinogenic photoreactive amino acid analogues with bicyclic structures containing a benzene ring “scaffold” (13), we set out to test such compounds as substrates for incorporation into a protein via PheRS-A294G. Other unnatural amino acids shown to be substrates for this aminoacyl tRNA synthetase variant display distinct yet limited structural divergence from phenylalanine. In contrast, the presently tested analogues, benzofuranylanine and benzotriazolylalanine (Fig. 1), contain an additional five-membered ring system fused to the benzene ring of phenylalanine. Although PheRS-\(\alpha\)A294G (and PheRS-\(\alpha\)T251G/A294G) appear rather flexible with respect to modifications at the \(\alpha\)aa position of the benzene side chain, it is far from obvious that this mutant would accommodate amino acids carrying double ring systems involving joint substitutions at the \(\alpha\)aa and \(\alpha\)aa position of the phenylalanine benzene side chain. Nevertheless, based on growth inhibition, we have previously proposed that benzofuranylanine is indeed a substrate for PheRS-A294G, opening up the possibility that such bicyclic amino acid analogs could be developed as substrates for protein synthesis (13).

We now present in vitro results showing that benzofuranylanine is a substrate for aminoacylation of tRNA\(^{\text{phe}}\) by PheRS-\(\alpha\)A294G and directly demonstrate the in vivo incorporation of this amino acid analogue into a protein using *E. coli* strains expressing mutant PheRS-\(\alpha\)-subunits and wild type tRNA\(^{\text{phe}}\). In addition, another photoreactive and structurally

\* This work was supported by grants from the Lundbeck Foundation (to T. B.), the Alfred Benzon Foundation (to M. I.), the Novo Nordisk Foundation (to M. I.), and the Danish Research Agency (to T. B., M. I., and P. E. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\† To whom correspondence should be addressed. E-mail: pen@imbg.ku.dk.

1 The abbreviations used are: PheRS, phenylalanine tRNA synthetase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; FAB-MS, fast atom bombardment mass spectroscopy; DHFR, dihydrofolate reductase; shtDHFR, short histidine tag DHFR.
related amino acid, benzotriazolylalanine, was synthesized and tested as a substrate for activation and aminoacylation.

EXPERIMENTAL PROCEDURES

Synthesis—All intermediate products have been obtained as mixtures of isomers (named as XZ) in a ratio of 1:1, as determined by high performance liquid chromatography. NMR data for XZ derivatives are given for two isomer products.

5-Hydroxymethyl-N-benzyl-benzotriazole (1XZ) — 5-Hydroxymethylbenzotriazole (14) (7.5 g, 50 mmol) and K₂CO₃ (4.0 g, 29 mmol) were suspended in acetonitrile (50 ml). Benzylbromide (10.2 g, 60 mmol) was added, and the mixture was stirred overnight at room temperature. The reaction mixture was evaporated to dryness in vacuo. The residue was dissolved in ethyl acetate and extracted with NaHCO₃ (saturated aqueous solution). The organic phase was dried over MgSO₄ and reduced in an oil. This oil was a mixture of three benzylation products at positions N1, N2, and N3 (Scheme 1). The isomers N1 and N3 were isolated as an inseparable mixture (5.5 g, 46%) by silica gel column chromatography eluting with ethyl acetate/n-hexane (2:1 v/v). FAB-MS m/z 240 (M+H) data: ¹H NMR (Me₂SO-d₆): δ 8.0–7.2 (m, 16H), 5.97 (s, 4H), 5.47 (t, J = 5.5 Hz), 4.67 (d, J = 5.5 Hz); ¹³C NMR (Me₂SO-d₆): δ 145.55, 138.93, 135.88, 131.90, 128.72, 127.98, 127.60, 127.48, 118.71, 115.89, 110.26, 107.11, 62.65, 50.95, 50.73.

2-Acetamino-2-carbethoxy-3-(N-benzyl-benzotriazole-5-yl)propanoic acid ethyl ester (3XZ) — Compound 1XZ (4.6 g, 19 mmol) was suspended in dichloromethane (100 ml). Phosphorous tribromide (5.5 g, 20 mmol) was added dropwise. After 4 h of stirring the reaction mixture was extracted with ice-cold water (2 x 100 ml) and subsequently with saturated aqueous NaHCO₃ (2 x 50 ml). The organic phase was dried over MgSO₄ and evaporated to dryness in vacuo. The remaining oil (5-bromomethyl-N-benzyl-benzotriazole, 2XZ) was added to a freshly made solution of sodium diethyl acetamidomalonate (prepared by treatment of diethyl acetamidomalonate (4.3 g, 20 mmol) with sodium hydride (60% dispersion in oil, 800 mg, 20 mmol) in dry tetrahydrofuran (100 ml) for 30 min and subsequent filtration of the salt and drying in vacuo for 30 min) in dry Me₂SO (10 ml) and stirred for 15 min at room temperature under nitrogen. The reaction mixture was taken into water (200 ml) and extracted with ethyl acetate (2 x 100 ml). The organic phase was dried over Na₂SO₄ and evaporated to dryness in vacuo. The residue was purified by silica gel column chromatography eluting with ethyl acetate/n-hexane (1:1 v/v), and 3XZ was obtained as colorless crystals (7.8 g, 89%). FAB-MS m/z 439 (M+H) data: ¹H NMR (CDCl₃): δ 7.9–6.9 (m, 16H), 6.48 and 6.38 (2s, 2H), 5.71 (s, 4H), 4.2–4.0 (m, 8H), 3.86 (s, 3H), 1.25 (s, 12H), 0.92 (s, 6H).

SCHEME 1. Steps during synthesis of benzotriazolylalanine.
3.70 and 3.67 (2s, 4H), 1.93 and 1.75 (2s, 6H), 1.2 (m, 12H); 13C NMR (CDCl₃): δ 169.11, 169.06, 167.05, 166.91, 145.60, 144.82, 135.35, 134.33, 134.10, 132.70, 131.89, 129.28, 128.83, 128.80, 128.36, 127.44, 126.117, 120.06, 119.25, 110.49, 109.40, 67.05, 66.85, 62.63, 62.55, 62.35, 52.27, 51.94, 37.77, 37.35, 22.82, 22.60, 13.80, 13.72.

N-Acetyl-3-(N-benzyl-benzotriazole-5-yl)-DL-alanine Ethyl Ester (4XZ) — Compound 3XZ (3.0 g, 6.8 mmol) and lithium chloride (2 g) were dissolved in a mixture of water (2 ml) and N,N-dimethylformamide (80 ml) and heated to 140 °C for 18 h. The solvent was then removed in vacuo, and the residue was taken up in water (50 ml). The aqueous phase was extracted with ethyl acetate (3 × 100 ml), and the combined organic phases were dried over Na₂SO₄ and evaporated to dryness in vacuo. The solid residue was purified by silica gel column chromatography, eluting with ethyl acetate.

4XZ was obtained as colorless crystals (2.3 g, 92%). FAB-MS m/z 367 (M+H) data: 1H NMR (CDCl₃): δ 7.9–7.0 (m, 16H), 6.3 and 6.2 (2d, 2H, J = 7.0 Hz), 5.8 (9, 4H), 4.7 (m, 2H), 3.5–3.2 (m, 4H), 2.0 and 1.9 (2s, 6H); 13C NMR (CDCl₃): δ 171.40, 171.30, 169.96, 168.99, 145.83, 144.96, 136.52, 134.61, 134.37, 132.98, 132.13, 129.54, 129.12, 128.69, 128.59, 127.72, 127.48, 126.29, 119.85, 110.05, 110.00, 61.79, 61.71, 61.59, 53.40, 53.24, 52.64, 41.53, 38.15, 37.88, 23.15, 23.07, 14.19.

N-Acetyl-3-(N-benzyl-benzotriazole-5-yl)-L-alanine (5XZ) — Compound 4XZ (2.2 g, 6 mmol) was dissolved in a mixture of Me₂SO (30 ml) and phosphate buffer (pH 7.5, 0.1 M, 50 ml). Carlsberg subtilisin (20 mg dry weight) was added, and the mixture was stirred overnight at room temperature. Water (50 ml) was added, and non-reacted ethyl ester (D-form) was removed by extraction with ethyl acetate (3 × 100 ml). The pH of the aqueous phase was adjusted to 2.5 by the addition of diluted HCl and extracted with ethyl acetate (4 × 100 ml). The organic phase was evaporated in vacuo to an oil, which was dissolved in water (100 ml) and freeze-dried. 5XZ was obtained as a white powder (840 mg, 40%).

FAB-MS m/z 339 (M+H) data: 1H NMR (CD₃OD): δ 7.9–7.2 (m, 16H), 5.9 (s, 4H), 4.7 (m, 2H), 3.4–3.0 (m, 4H), 1.9 and 1.7 (m, 6H); 13C NMR (CD₃OD): δ 171.40, 171.10, 169.89, 145.83, 144.96, 134.70, 136.70, 136.26, 134.95, 133.95, 132.60, 130.47, 130.46, 130.37, 130.34, 130.10, 129.57, 128.98, 127.38, 120.91, 112.51, 55.16, 55.18, 52.30, 41.54, 37.50, 37.20, 23.16, 23.07.

L-3-(N-Benzyl-benzotriazole-5-yl)-L-alanine Hydrochloride (6XZ) — Compound 5XZ (700 mg, 2 mmol) was dissolved in 5 N HCl (50 ml) and heated under reflux for 4 h. After cooling, the solvent was removed in vacuo, and the solid residue was dissolved in tetrahydrofuran (5 ml). This solution was drained into diethyl ether (50 ml), and 6XZ was collected as a white powder by filtration (650 mg, 93%).

FAB-MS m/z 297 (M+H) data: 1H NMR (CD₃OD): δ 7.9–7.2 (m, 16H), 5.9 (s, 4H), 4.7 (m, 2H), 3.5–3.2 (m, 4H); 13C NMR (Me₂SO-d₆): δ 171.10, 145.85, 144.95, 136.70, 136.26, 134.77, 133.95, 132.60, 130.47, 130.10, 129.57, 128.98, 127.38, 120.91, 120.77, 112.51, 55.16, 55.18, 52.30, 37.40, 37.20.

FIG. 2. UV spectra and photolability of benzofuranylalanine. A–C, irradiation at increasing wavelengths as indicated. Left panel, selected spectra from the irradiation time course. Right panel, UV absorbance at the indicated wavelength as a function of irradiation time. The benzofuranylalanine concentrations used were 0.2 mM (A) and 5 mM (B and C).
L-3-(Benzotriazole-5-yl)-L-alanine Hydrochloride (7)—Compound 6XZ (600 mg, 2 mmol) was dissolved in glacial acetic acid (5 ml) and hydrogenated at 5.5 atm in the presence of catalytic amounts of Pd on charcoal for 48 h. The catalyst was removed by filtration, and the filtrate evaporated to dryness in vacuo. The residue was triturated with diethyl ether, and the product was obtained as a white powder (400 mg, 96%). FAB-MS m/z data: 1H NMR (CD3OD): 8.05 (m, 2H), 7.74 (d, 1H, J = 9.1 Hz), 4.47 (t, 1H, J = 6.5 Hz), 3.7-3.4 (ABX-system, J\(_{\text{AB}}\) = 17.5 Hz, J\(_{\text{AX}}\) = 6.0 Hz, J\(_{\text{BX}}\) = 7.2 Hz); 13C NMR (CD3OD): 161.20, 128.26, 127.84, 127.26, 122.36, 122.15, 121.39, 106.40, 45.35. High resolution mass spectrometry results for C9H11N4O2 were 207.0882 (calculated) and 207.0881 (experimental).

UV Irradiation—Aqueous solutions of benzofuranylalanine and benzotriazolylalanine at the indicated concentrations in a quartz cuvette sealed with Parafilm were placed immediately adjacent to the desired UV light source with the following characteristics: Struers UVG-11, 254 nm; Philips TL20W/12RS, 320 nm; Phillips 20 W/09N, 365 nm. Samples were irradiated at room temperature for the desired length of time. At the desired time intervals the cuvette was removed, and the UV absorption spectrum was recorded using a Cary 300 Bio UV-visible spectrophotometer (Varian).

Charging and Competition Analyses—Wild type and aA294G E. coli PheRS were purified as previously described (10). E. coli tRNA\(^{\text{Phe}}\) was from Sigma. Pyrophosphate (PP\(_i\)) exchange and aminoacylation reactions were performed as described (10), with unlabeled benzofuranylalanine included at 2 and 4 mM for the determination of inhibition constants during aminoacylation. The direct attachment of benzofuranylalanine and benzotriazolylalanine to in vitro transcribed tRNA was monitored by direct \(^{32}\)P labeling of tRNA\(^{\text{Phe}}\) using E. coli tRNA-terminal nucleotidyltransferase (15) followed by aminoacylation and product visualization as previously described (16).

Expression Analyses—In vivo incorporation of benzofuranylalanine into recombinant murine dihydrofolate reductase (DHFR) was performed as previously described using the phenylalanine auxotrophic
The reaction contained 100 mM Hepes, pH 7.2, 10 mM MgCl₂, 30 mM KCl, 2 mM ATP, 1 µM E. coli tRNA^{Awe} (Roche Applied Science), and a trace (3.5 nCi) of ³²P-labeled tRNA^{Awe}, and aminoacylation was performed at 1 µM tRNA and 1 nm wild type PheRS (diamonds and squares) or α-A294G mutant (triangles and circles) with 2 µM Phe (diamonds and triangles) or 0.2 µM benzofuranylalanine (squares and circles).

strains AF-1Q/pQE-15 (PheRS α wild type) and AF-1Q/pQE-FS (PheRS-αA294G mutant) (11). An overnight culture was diluted into fresh M9 minimal media supplemented with the 20 amino acids at 20 µg/ml and the antibiotics ampicillin (100 µg/ml) and chloramphenicol (40 µg/ml) and grown to an A₆₀₀ of 0.6–1.0. The cells were harvested by centrifugation, washed twice in 20 ml of ice-cold 0.9% NaCl, and resuspended in fresh M9 media containing antibiotics and all natural amino acids except phenylalanine. The cells were separated into aliquots of 1–2 ml fractions and supplemented with benzofuranylalanine (2 mM) and phenylalanine (0.1 mM) as indicated, grown for 10 min at 37 °C, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and then grown for 4–5 h at 30 °C. 100 µl of cells grown in the presence of phenylalanine (and the A₂₉₄₀/ml normalized amount of cells grown with unnatural amino acid) were harvested by centrifugation. The pellet was resuspended in 20 µl of B-PER (Pierce) and 20 µl of 2x SDS loading buffer and heated to 90 °C for 5–10 min, and 10 µl was analyzed using 12% acrylamide-SDS page minigels followed by Coomasie Brilliant Blue staining.

Protein Purification—Protein purification was done using strain AF-1Q/pQE-15 (PheRS-αA294G mutant) from 10 ml of M9 minimal media cultures supplemented with 19 amino acids (20 µg/ml) and benzofuranylalanine (2 mM) or phenylalanine (0.1 mM) as described (3) and using nickel nitritotriacetic acid chromatography spin columns under denaturing conditions as recommended (Qiagen). Buffer exchange from 8 M urea, 100 mM sodium phosphate, pH 4.5, into water was done using 2 ml Amicon 10-kDa cutoff ultrafiltration spin filters.

Mass Spectrometry—Mass spectrometry analyses were performed using a SpectraChrom Kompact MALDI II instrument from Kratos. MALDI-TOF samples were prepared by mixing 1 µl of protein sample (0.2–0.25 µg) per 4 µl of matrix (e-cyano-4-hydroxycinnamic acid at 10 mg/ml in 50% acetonitrile 50% H₂O). The samples were spotted onto MALDI-TOF slides using 4.5 µl per application. Chymotrypsinogen A (25,657 Da) and β-lactoglobulin A (18,384 Da) were used as calibration standards. The slide was examined for “sweet spots” before running the experiment. 250 profiles were accumulated per sample.

RESULTS AND DISCUSSION

Synthesis of Bicyclic Amino Acids—Benzofuranylalanine was synthesized as previously reported (13), and the novel benzotriazoleallylalanine was prepared as shown in Scheme 1 starting from commercially available 5-(hydroxymethyl)triazole using standard chemistry. Enantiomeric resolution was achieved using Carlsberg subtilisin (13) for specific hydrolysis of the L-form of an ester intermediate.

Photochemistry of the Unnatural Amino Acids—The [2+2] photocycloadditions of alkenes are extremely specific and often of high (quantum) yields. These could therefore be very useful for sequence-specific post-modification of proteins, provided an activated alkene with appropriate absorbance characteristics

can be site specifically incorporated into a protein, since no other functionality of similar reactivity is naturally present in proteins.

Benzofurans undergo [2+2] photocycloaddition with a variety of alkenes (e.g. Ref. 17), although the furan double bond is only weakly activated by the adjacent oxygen. However, benzofurans also undergo other photochemical reactions. To study the photosensitivity of benzofuranylalanine, the compound was irradiated at different wavelengths, and the corresponding UV absorption spectra were recorded over time (Fig. 2). Upon irradiation at 254 nm the absorption peak at 245 nm declined, and a “shoulder” peak with a maximum of 342 nm appeared with similar kinetics (estimated t½ = 50 min) compatible with a photochemical reaction (Fig. 2A). However, because most proteins (due to the presence of aromatic amino acids) and all nucleic acids as well as other cell components have strong absorption at 254 nm, many unwanted side reactions are bound to occur both in vitro and especially in vivo upon such irradiation. Conversely, considerably fewer photoreactions take place in biological systems upon irradiation at longer wavelengths. Consequently, we also irradiated the amino acids at 320 and 365 nm, where the benzofuran chromophore exhibits a weak absorption (λ₃₂₀ = 342 nm), now focusing on the UV spectra in the range 300–400 nm (Fig. 2, B and C). At 320 nm of irradiation, both the spectral changes as well as the time course of the change in absorbance at the initial absorbance maximum of 342 nm (Fig. 2B) clearly show that at least two photochemical transformations take place, a fast reaction with an estimated t½ of ~ 6 min and a subsequent slow reaction over many hours. Strikingly, at 365 nm of UVA irradiation only the fast component was observed. We have not attempted to analyze the actual products at this stage, but these results clearly demonstrate the photosensitivity of the benzofuran ligand, and thus, its potential utility as a photochemical handle upon incorporation into proteins. A potential caveat of using UVA irradiation (λ, ~335 nm) involves the formation of photo adducts between thiouridine and cytidine at positions 8 and 13 in some tRNAs of E. coli (18), causing an amino acid starvation response. Once the cells are removed from UV exposure, however, the effects are reversed, and they recover by synthesis of new functional tRNAs (19).

Benzotriazoles undergo photochemical reactions reminiscent of the highly photosensitive azides. The predominant photoreaction involves release of molecular nitrogen (N₂) and formation of a radical species that may either undergo radical insertion reactions or nucleophilic addition reactions (e.g. Ref. 20), both giving rise to chemical cross-linking. Upon incorporation
Mass spectrometry analysis of benzofuranylalanine incorporation into shtDHFR. Shown are examples of mass spectra showing shtDHFR expressed in the presence of benzofuranylalanine (A) and phenylalanine (B). The molecular masses obtained for this particular experiment are indicated. Protein mass determination was calculated as the mean of 4–5 experiment repetitions. The molecular weights obtained \( \pm \) S.D. is 24,284 \( \pm \) 119 (shtDHFR containing benzofuran) and 23,957 \( \pm \) 76 (shtDHFR-Phe), and these masses are statistically significantly different (double-sided Student's t test for data with equal variance, \( p < 0.005; n = 9 \)). The calculated mass of shtDHFR-Phe is 24040. Sequencing of the shtDHFR-coding region revealed no amino acid alterations as compared with the published sequence (Qiagen, plasmid pQE15). The discrepancy of the found and calculated masses of shtDHFR-Phe are therefore ascribed to experimental inaccuracy.

Fig. 6. Mass spectrometry analysis of benzofuranylalanine incorporation into shtDHFR. Shown are examples of mass spectra showing shtDHFR expressed in the presence of benzofuranylalanine (A) and phenylalanine (B). The molecular masses obtained for this particular experiment are indicated. Protein mass determination was calculated as the mean of 4–5 experiment repetitions. The molecular weights obtained \( \pm \) S.D. is 24,284 \( \pm \) 119 (shtDHFR containing benzofuran) and 23,957 \( \pm \) 76 (shtDHFR-Phe), and these masses are statistically significantly different (double-sided Student's t test for data with equal variance, \( p < 0.005; n = 9 \)). The calculated mass of shtDHFR-Phe is 24040. Sequencing of the shtDHFR-coding region revealed no amino acid alterations as compared with the published sequence (Qiagen, plasmid pQE15). The discrepancy of the found and calculated masses of shtDHFR-Phe are therefore ascribed to experimental inaccuracy.

These results are consistent with the kinetic analyses and confirm that benzofuranylalanine, but not benzotriazolylalanine, can be attached to tRNA\(^{\text{Phe}}\) by PheRS-\(\Delta A294G\). In addition, they indicate that benzofuranylalanine, in contrast to natural non-cognate amino acids, is not a substrate for the hydrolytic proofreading activity of PheRS (10).

In Vivo Incorporation of Bicyclic Amino Acids—To test whether benzofuranylalanine and/or benzotriazolylalanine are in vivo protein synthesis substrates, we used a phenylalanine auxotrophic E. coli strain encoding PheRS-\(\Delta A294G\) on an episome (strain AF-1Q/pQE-FS) and the isogenic strain lacking the mutant tRNA synthetase-encoding gene (strain AF-1Q/pQE-15) as a control. Both strains harbor an isopropyl-1-thio-\(\beta\)-D-galactopyranoside-inducible expression cassette encoding murine DHFR carrying a short histidine tag (shtDHFR). As previously reported (11), both strains show abundant overproduction of shtDHFR upon the addition of isopropyl-1-thio-\(\beta\)-D-galactopyranoside in the presence of phenylalanine (Fig. 5, compare lanes 1 with 2 and lane 5 with 6). However, only strain AF-1Q/pQE-FS produced large amounts of shtDHFR when substituting benzofuranylalanine for phenylalanine (compare lanes 3 and 7). In fact, in the presence of wild type PheRS alone shtDHFR production did not increase beyond background when conducting the experiment with benzofuranylalanine. These results indicate that benzofuranylalanine is indeed an in vivo substrate for PheRS-\(\Delta A294G\) and, consistent with the in vitro data, is not utilized by wild type PheRS. A slight but notable expression of shtDHFR was observed even in the absence of added phenylalanine or benzofuranylalanine (lanes 4 and 8). This was previously ascribed to residual cellular pools of phenylalanine remaining even after extensive washing of the cells (3, 11). Similar analyses were conducted using the benzotriazolylalanine. Consistent with the in vitro data, no shtDHFR production above background was observed upon the addition of benzotriazolylalanine (data not shown). We also tested the strain AF-1Q/pQE-T251G/A294G carrying a double mutant PheRS \(\alpha\)-subunit (3) for incorporation of unnatural amino acids.
into shtDHFR. In accordance with the data obtained using strain AF-1Q/pQE-FS, the double mutant utilized benzofuran
ylalanine but showed no shtDHFR expression above back-
ground in the presence of benzotriazolylalanine (data now
shown). Thus, benzofuranylalanine, but not benzotriazolylala-
nine, is a substrate for PheRS ρ165), incorporation of benzofuran into the
reporter protein should be readily detectable as a significant
mass increase of shtDHFR containing benzofuran. Mass spect-
rometry analysis of purified protein revealed molecular
masses of 24,284 and 23,957 Da for shtDHFR expressed in the
presence of benzofuranylalanine and phenylalanine, respec-
respectively (Fig. 6). This clearly demonstrates the incorpora-
tion of unnatural amino acid and corresponds to an average of ~8
benzofuranylalanine replacements in nine possible positions.

Conclusions—The data presented show that benzofuran-
ylalanine, but not benzotriazolylalanine, is an efficient substrate
for cellular protein synthesis as a result of its ability to be
attached to tRNAPhe by PheRS

Analysis of Benzofuran-containing shtDHFR—Because ben-
zo furanylalanine (M, 205) has a larger mass as compared with
phenylalanine (M, 165), incorporation of benzofuran into the
tRNAPhe-derived suppressor species or perhaps another pair
of cellular protein synthesis as a result of its ability to be
attached to tRNAPhe by PheRS

REFERENCES
1. Magliery, T. J., Pastronak, M., Anderson, J. C., Santoro, S. W., Herberich, B.,
Meggere, E., Wang, L., and Schultz, P. G. (2003) in Translational Mecha-
nism (Lapointe, J., and Brakier-Gingras, L., eds) Kluwer Academic Pub-
lisbers, Norwell, MA/Plenum Publishing Corp., New York
2. Ibba, M., and Hennecke, H. (1995) FEBS Lett. 364, 272–275
3. Datta, D., Wang, P., Carrico, I. S., Mayo, S. L., and Tirrell, D. A. (2002) J. Am.
Chem. Soc. 124, 5652–5653
4. Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001) Science 292,
498–500
5. Zhang, Z., Gildersleeve, J., Yang, Y. Y., Xu, R., Loo, J. A., Uryu, S., Kong,
C. H., and Schultz, P. G. (2004) Science 303, 371–373
6. Kohner, C., Xie, L., Kelleer, S., Varshney, U., and RajBhandary, U. L. (2001)
Proc. Natl. Acad. Sci. U. S. A. 98, 14310–14315
7. Kowal, A. K., Kohner, C., and RajBhandary, U. L. (2001) Proc. Natl. Acad.
Sci. U. S. A. 98, 2268–2273
8. Chin, J. W., Cropp, T. A., Anderson, J. C., Mukherji, M., Zhang, Z., and
Schultz, P. G. (2003) Science 301, 964–967
9. Ibba, M., and Soll, D. (2000) Annu. Rev. Biochem. 69, 617–650
10. Ibba, M., Kast, P., and Hennecke, H. (1994) Biochemistry 33, 7107–7112
11. Sharma, N., Furter, R., Kast, P., and Tirrell, D. A. (2000) FEBS Lett. 467,
37–40
12. Kirchenbaum, K., Carrico, I. S., and Tirrell, D. A. (2002) Chembiochem 3,
235–237
13. Behrens, C., Nielsen, J. N., Fan, X. J., Daoi, X., Kim, K. H., Praetorius-Ibba,
M., Nielsen, P. E., and Ibba, M. (2000) Tetrahedron 56, 9443–9449
14. Katritzky, A. R., Ji, F. B., Fan, W. Q., and Delprato, I. (1993) Synth. Commun.
23, 2019–2025
15. Seth, M., Thurlow, D. L., and Hsu, Y. M. (2002) Biochemistry 41, 4521–4532
16. Wollson, A. D., and Uhlenbeck, O. C. (2002) Proc. Natl. Acad. Sci. U. S. A.
99, 5965–5970
17. Sakamoto, M., Kinhara, A., Yagi, T., Mine, T., Yamaguchi, K., and Fujita, T.
(2000) Chem. Commun. 1201–1202
18. Yaniv, M., Favre, A., and Barrell B. G. (1969) Nature 223, 1331–1333
19. Ramabhadran, T. V., and Jagger, J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73,
59–63
20. Wang, H., Burda, C., Persy, G., and Wirz, J. (2000) J. Am. Chem. Soc. 122,
5849–5855
21. Kwon, I., Kirchenbaum, K., and Tirrell, D. A. (2003) J. Am. Chem. Soc. 125,
7512–7513
22. Furter, R. (1998) Protein Sci. 7, 419–426
