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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 benzofuranylalanine and benzotriazolylalanine were synthesized, and their photolability was tested by UV irradiation at 254, 320, and 365 nm. Although both showed photo reactivity, benzofuranylalanine 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 $tRNA^{Phe}$ and for protein mutagenesis via the phenylalanyl-tRNA synthetase variant αA294G that is able to facilitate *in vivo* protein synthesis using a range of *para*-substituted phenylalanine analogues. The results demonstrate that benzofuranylalanine, but not benzotriazolylalanine, is a substrate for phenylalanine tRNA synthetase αA294G, 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 synthetases or of the tRNA-aminoacyl tRNA synthetase pair. The aminoacyl tRNA synthetases charge tRNA with cognate amino acid before delivery of the aminoacyl-tRNA by elongation factor Tu to the ribosome for incorporation into nascent polypeptide (9). One approach to the development of unnatural amino acid recognition involves selective mutagenesis of the relevant aminoacyl tRNA synthetase to recognize amino acid analogues that are close structural mimics of the cognate amino acid (2, 3). For instance, an *E. coli* phenylalanine tRNA synthetase (PheRS)[1] variant carrying a single Ala → Gly amino acid substitution at α-subunit residue 294 (PheRS-αA294G) (10) displays relaxed substrate specificity *in vivo* toward a number of *para*-substituted phenylalanine mimics. Examples include several halogenated phenylalanines including *p*-chlorophenylalanine (2), *p*-bromophenylalanine (11) and *p*-iodophenylalanine as well as *p*-cyanophenylalanine, *p*-ethynylphenylalanine, *p*-azidophenylalanine, and 2-,3-, and 4-pyridylalanine (12). Introducing the additional mutation Thr → Gly in position 251 (PheRS-αT251G/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-αA294G. 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, benzofuranylalanine and benzotriazolylalanine (Fig. 1), contain an additional five-membered ring system fused to the benzene ring of phenylalanine. Although PheRS-αA294G (and PheRS-αT251G/A294G) appear rather flexible with respect to modifications at the *para* 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 *meta* and *para* position of the phenylalanine benzene side chain. Nevertheless, based on growth inhibition, we have previously proposed that benzofuranylalanine is indeed a substrate for PheRS-αA294G, 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 benzofuranylalanine is a substrate for aminoacylation of $tRNA^{Phe}$ by PheRS-αA294G and directly demonstrate the *in vivo* incorporation of this amino acid analogue into a model protein using *E. coli* strains expressing mutant PheRS α-subunits and wild type $tRNA^{Phe}$. In addition, another photoreactive and structurally

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[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-Hydroxymethyl-benzotriazole (14) (7.5 g, 50 mmol) and K_2CO_3 (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 $NaHCO_3$ (saturated aqueous solution). The organic phase was dried over $MgSO_4$ 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 unseparable 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: ^{1}H NMR (Me_2SO-*d*6): δ 8.0–7.2 (m, 16H), 5.97 (s, 4H), 5.47 (t, 2H, J = 5.5 Hz), 4.67 (d, 4H, J = 5.5 Hz); ^{13}C NMR (Me_2SO-*d*6): δ 145.55, 138.93, 135.88, 131.90, 128.72, 127.98, 127.60, 127.48, 126.90, 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 × 100 ml) and subsequently with saturated aqueous $NaHCO_3$ (2 × 50 ml). The organic phase was dried over $MgSO_4$ 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 Me_2SO (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 × 100 ml). The organic phase was dried over Na_2SO_4 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: ^{1}H NMR ($CDCl_3$): δ 7.9–6.9 (m, 16H), 6.48 and 6.38 (2s, 2H), 5.71 (s, 4H), 4.2-4.0 (m, 8H),

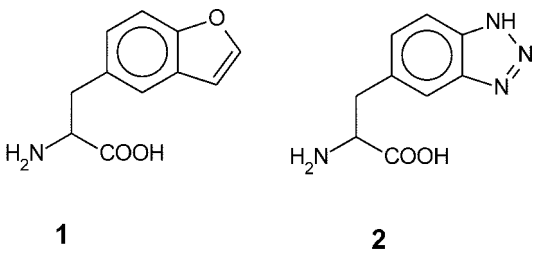
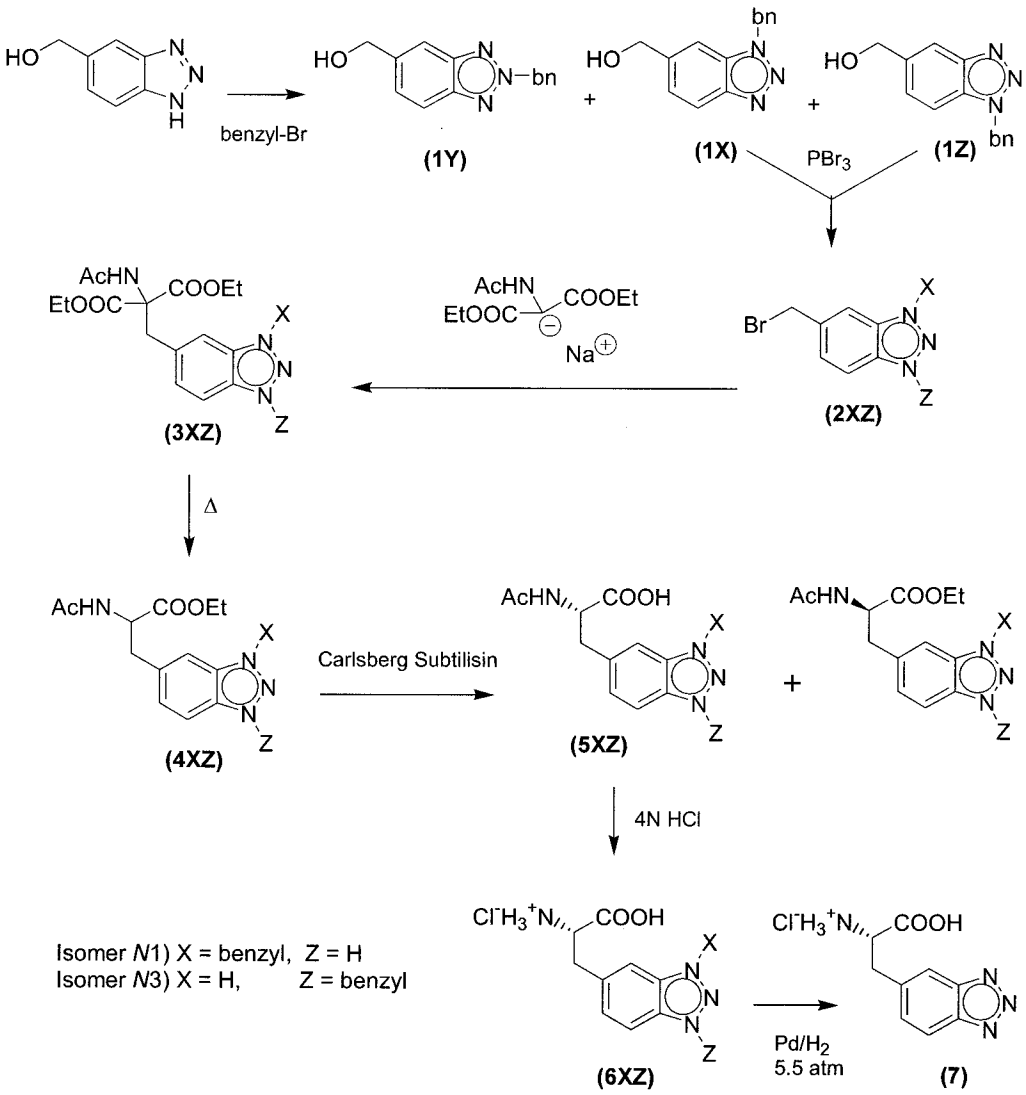


FIG. 1. **Chemical structure of the photoreactive amino acid analogues used in this study.** *1*, benzofuranylalanine; *2*, benzotriazolylalanine.



SCHEME 1. **Steps during synthesis of benzotriazolylalanine.**

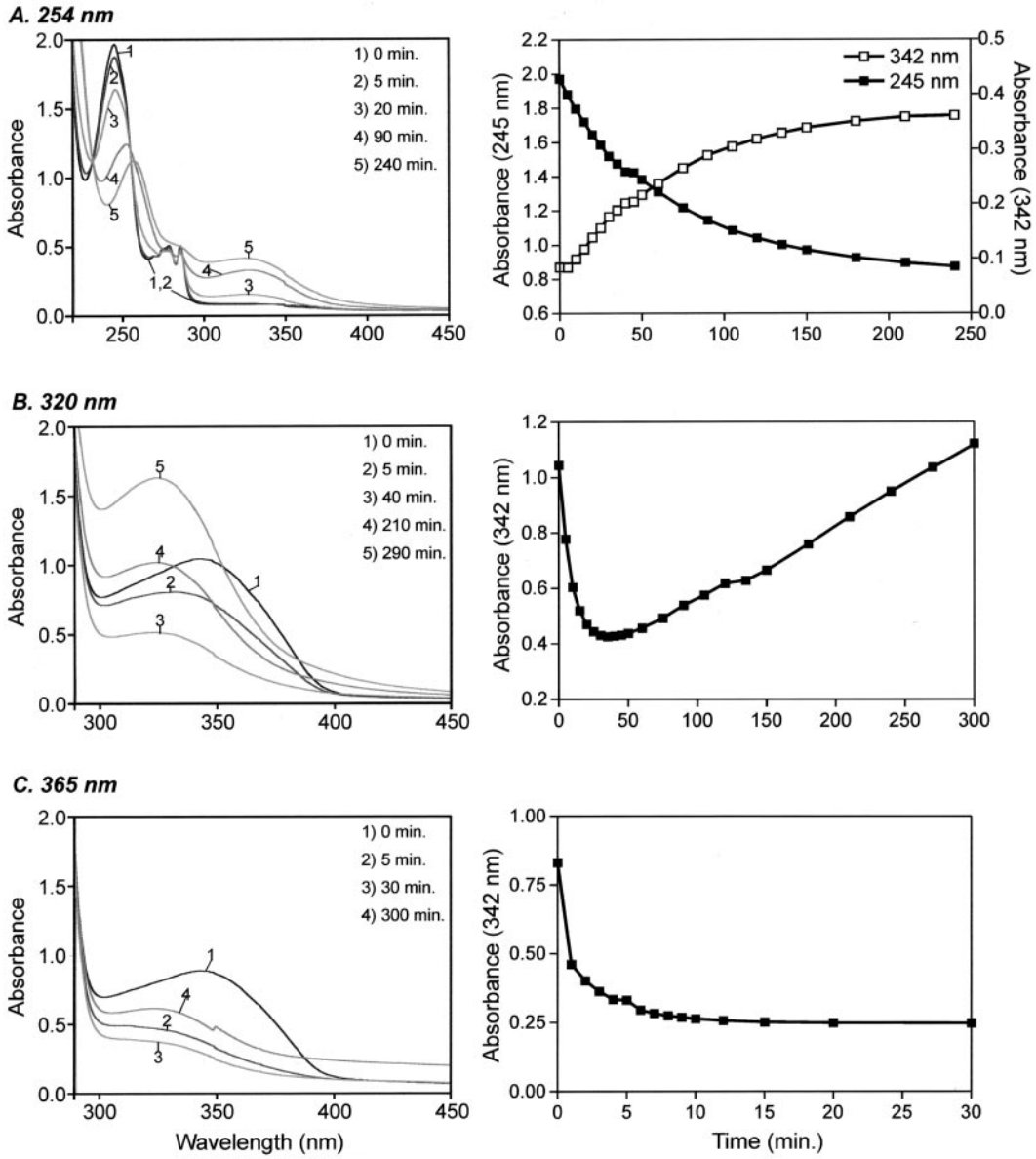


FIG. 2. **UV spectra and photolability of benzofuranylalanine.** *A–C*, irradiation at increasing wavelengths as indicated. *Left panel*, selected spectra from the irradiation time course. *Right panel*, UV absorbance at the indicated wavelength as a function of irradiation time. The benzofuranylalanine concentrations used were 0.2 mM (*A*) and 5 mM (*B* and *C*).

3.70 and 3.67 (2s, 4H), 1.93 and 1.75 (2s, 6H), 1.2 (m, 12H); ^{13}C NMR ($CDCl_3$): δ 169.11, 169.06, 167.05, 166.91, 145.60, 144.82, 135.35, 134.33, 134.10, 132.70, 131.89, 131.66, 129.79, 129.28, 128.83, 128.80, 128.36, 128.25, 127.44, 126.92, 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 Na_2SO_4 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: ^{1}H NMR ($CDCl_3$): δ 7.9–7.0 (m, 16H), 6.3 and 6.2 (2d, 2H, J = 7.0 Hz), 5.81 (s, 4H), 4.95 (m, 2H), 4.2-4.0 (m, 4H), 3.2 (m, 4H), 2.0 and 1.9 (2s, 6H), 1.2–1.0 (m, 6H); ^{13}C NMR ($CDCl_3$): δ 171.40, 171.30, 169.96, 169.89, 145.83, 144.96, 136.52, 134.61, 134.37, 133.10, 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, 52.30, 41.53, 38.15, 37.88, 23.15, 23.07, 14.19.

*LN-Acetyl-3-(N-benzyl-benzotriazole-5-yl)-L-alanine (**5XZ**)*—Compound **4XZ** (2.2 g, 6 mmol) was dissolved in a mixture of Me_2SO (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: ^{1}H NMR (CD_3OD): 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); ^{13}C NMR (CD_3OD): δ 171.40, 171.10, 169.89, 145.83, 144.96, 136.70, 136.26, 134.77, 133.95, 132.60, 130.47, 130.10, 129.57, 128.98, 127.38, 120.91, 112.51, 52.17, 53.19, 52.98, 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: ^{1}H NMR (CD_3OD):δ 7.9–7.2 (m, 16H), 5.9 (s, 4H), 4.7 (m, 2H), 3.5–3.2 (m, 4H); ^{13}C NMR (Me_2SO-*d*6): δ 171.10, 145.85, 144.95, 136.70, 136.26, 134.77, 133.95, 132.60, 130.46, 130.10, 129.57, 128.98, 127.38, 120.91, 120.77, 112.51, 55.16, 55.18, 52.98, 50.42, 37.49, 37.20.

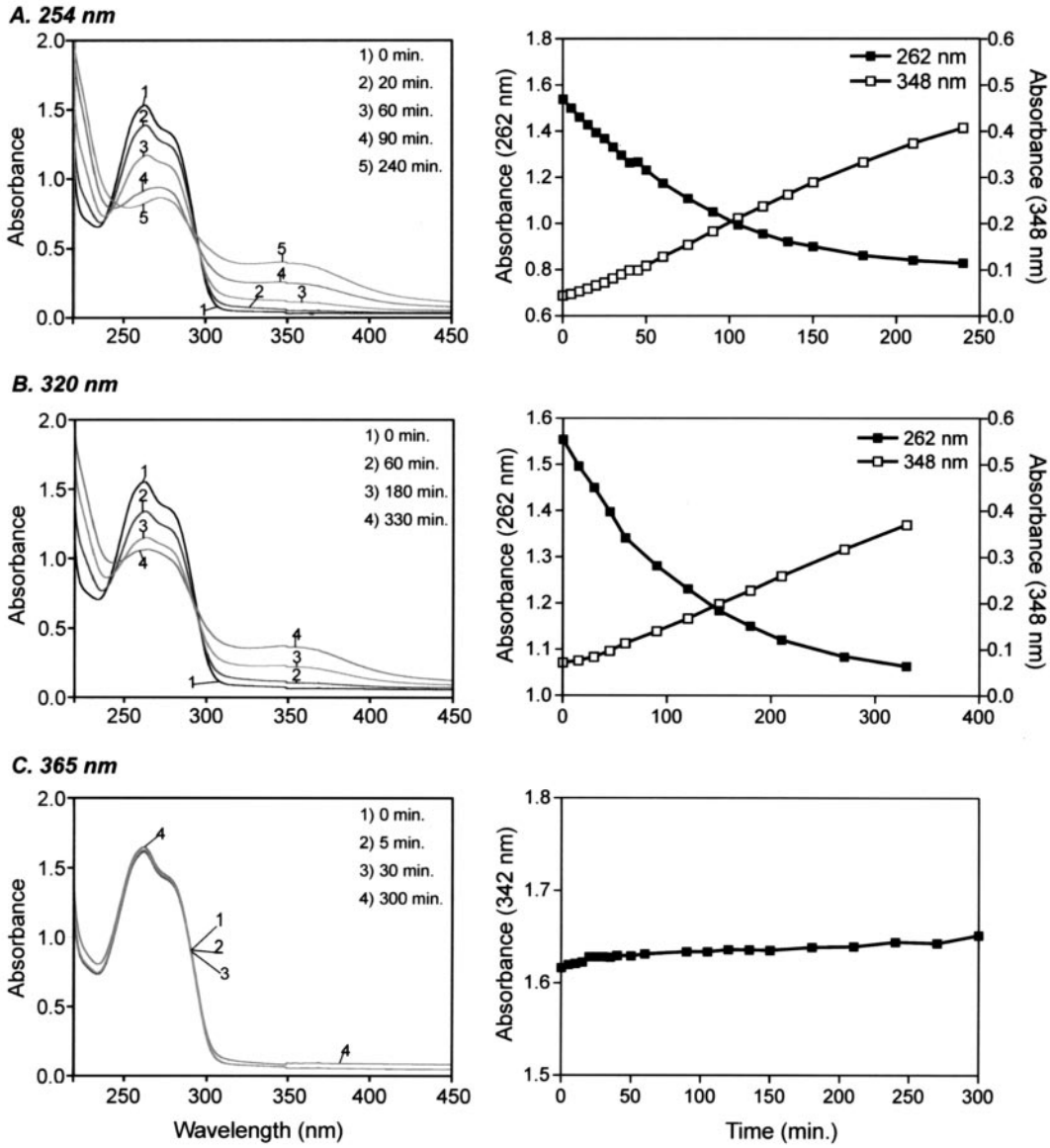


FIG. 3. **UV spectra and photosensitivity of the benzotriazolylalanine.** *A–C*, irradiation at different wavelengths as indicated. *Left panel*, selected spectra from the irradiation time course. *Right panel*, UV absorbance at the indicated wavelength as a function of irradiation time. A benzotriazolylalanine concentration of 0.4 mM was used throughout.

*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* 207 (M+H) data: ^{1}H NMR (CD_3OD): δ 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_{AB} = 17.5 Hz, J_{AX} = 6.0 Hz, J_{BX} = 7.2 Hz); ^{13}C NMR (CD_3OD): δ 161.20, 128.26, 127.84, 127.26, 122.36, 122.15, 121.39, 106.40, 45.35. High resolution mass spectrometry results for $C_9H_{11}N_4O_2$ 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 αA294G *E. coli* PheRS were purified as previously described (10). *E. coli* $tRNA^{Phe}$ 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 $tRNA^{Phe}$ using *E. coli* tRNA-terminal nucleotidyltransferase (15) followed by aminoacylation and product visualization as previously described (16).

TABLE I
Steady-state kinetic parameters for aminoacylation by αA294G PheRS with [^{14}C]-phenylalanine in the presence of [^{12}C]-benzofuranylalanine (Bzf)

Bzf	K_m	k_{cat}	K_i[a]
mM	*μM Phe*	*min^{-1}*	*μM Bzf*
0	20.2 ± 6	211 ± 27	NA
2	159 ± 39	179 ± 24	291 ± 110
4	333 ± 100	163 ± 26	258 ± 110

[a] K_i was determined from the following formula $K_{Mapp} = K_{Mreal}$ (1 + [Bzf]/K_i). Phe, phenylalanine; NA, not applicable.

Expression Analyses—In vivo incorporation of benzofuranylalanine into recombinant murine dihydrofolate reductase (DHFR) was performed as previously described using the phenylalanine auxotrophic

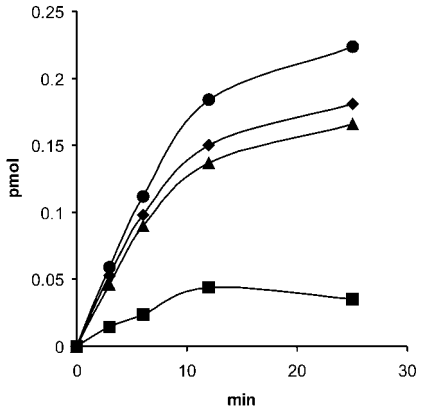


FIG. 4. **PheRS-αA294G aminoacylates tRNA^{Phe} with benzofuranylalanine.** tRNA^{Phe} was labeled as previously described (16). The reaction contained 100 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 30 mM KCl, 2 mM ATP, 1 μM *E. coli* tRNA^{Phe} (Roche Applied Science), and a trace (3.5 nCi) of 3′ ^{32}P-labeled tRNA^{Phe}, and aminoacylation was performed at 1 μM tRNA and 1 nM wild type PheRS (*diamonds* and *squares*) or αA294G mutant (*triangles* and *circles*) with 2 mM Phe (*diamonds* and *triangles*) or 0.2 mM benzofuranylalanine (*squares* and *circles*).

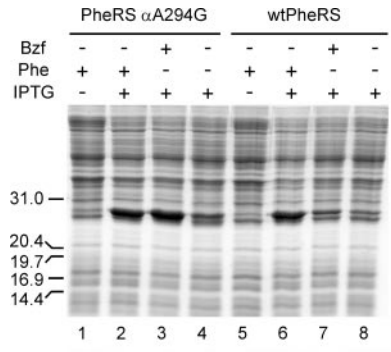


FIG. 5. **Expression analysis of benzofuranylalanine incorporation by PheRS-αA294G.** Analysis of total protein content from AF-1Q/pQE-FS (*lanes 1–4*) or AF-1Q/pQE-15 (*lanes 5–8*). The cells were incubated as described under "Experimental Procedures" with the indicated amino acids. Positions for a protein molecular weight marker are indicated on the *left side* of the gel. *Bzf*, benzofuranylalanine; *wt*, wild type.

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 A_{600} 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 A_{600}/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 Coomassie Brilliant Blue staining.

Protein Purification—Protein purification was done using strain AF-1Q/pQE-FS (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 nitrilotriacetic 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 (α-cyano-4 hydroxycinnamic acid at 10 mg/ml in 50% acetonitrile 50% H_2O). The samples were spotted onto MALDI-TOF slides using 4.5 μl per application. Chymotrypsinogen A (25,657 Da) and β-lactoglobulin A (18,364 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 benzotriazolylalanine 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 $t_{½}$, ~50 min) compatible with a photochemical reaction (Fig. 2*A*). 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 (λ_{max} = 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. 2*B*) clearly show that at least two photochemical transformations take place, a fast reaction with an estimated $t_{½}$ 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 (N_2) 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

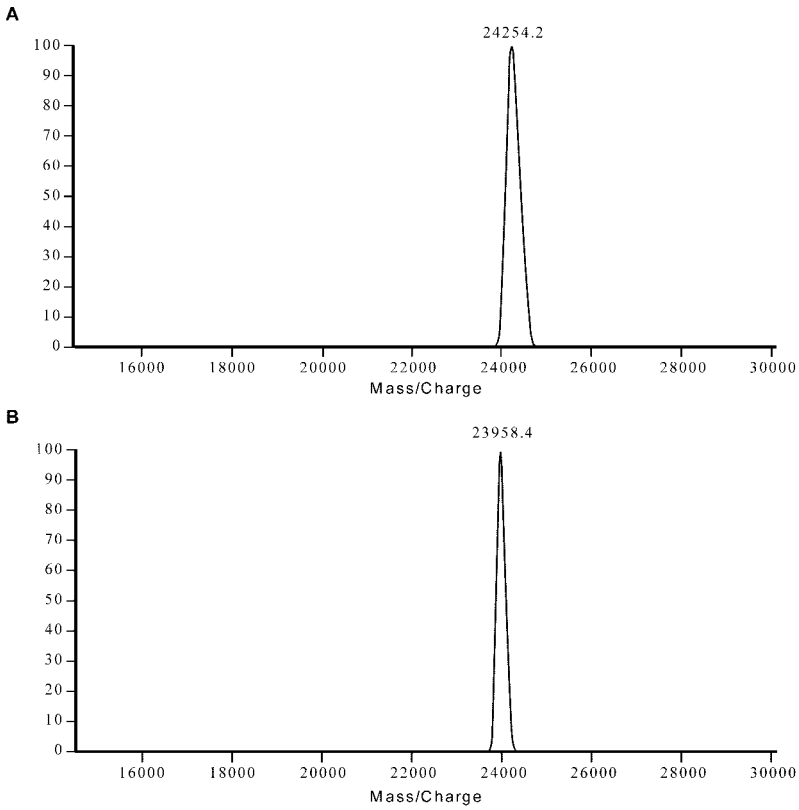


FIG. 6. **Mass spectrometry analysis of benzofuranylalanine incorporation into shtDHFR.** Shown are examples of mass spectra showing shtDHFR expressed in the presence of benzofuranylalanine (*A*) and phenylalanine (*B*). The molecular masses obtained for this particular experiment are indicated. Protein mass determination was calculated as the mean of 4–5 experiment repetitions. The molecular weights obtained ± S.D. is 24,284 ± 119 (shtDHFR containing benzofuran) and 23,957 ± 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.

into a protein, the benzotriazole ligand would constitute a site for specific photochemical cross-linking to an interacting biological ligand, *e.g.* another protein or a nucleic acid.

Irradiation at 254 and 320 nm of the benzotriazolylalanine resulted in pronounced spectral changes, fully compatible with a single photochemical conversion taking place (isosbestic points are observed at least at shorter irradiation times), with an estimated $t_{½}$ of 60 min (Fig. 3, *A* and *B*). These results support the potential use of this amino acid for site-specific photosensitization of proteins if incorporated. However, at 365 nm of irradiation (at which wavelength the benzotriazole does not show any significant absorption) no spectral changes were observed, indicating that this wavelength cannot be utilized for induction of photochemical reactions using the benzotriazolylalanine.

Steady-state Kinetics for Bicyclic Amino Acids—To investigate charging with unnatural amino acids, we first tested the ability of benzofuranylalanine and benzotriazolylalanine to inhibit aminoacylation of $tRNA^{Phe}$. Benzofuranylalanine did not show any inhibition of the wild type enzyme (data not shown) but acted as a competitive inhibitor for PheRS-αA294G (Table I). In contrast, the addition of benzotriazolylalanine had no effect on aminoacylation by either form of PheRS. To further investigate whether benzofuranylalanine might be a substrate for activation by the mutant form of the enzyme, PP_i exchange analysis was performed. The PP_i exchange reaction showed that benzofuranylalanine is a substrate for PheRS-αA294G, with a K_m of 2.3 ± 0.4 mM and a k_{cat} of 923 ± 65 min^{-1}. PP_i exchange with the wild type enzyme showed some low but inconsistent activity, suggesting that benzofuranylalanine might be poorly activated. As expected, phenylalanine showed a higher reaction rate, and no activity at all was seen when the reaction was performed without substrate.

We also tested benzofuranylalanine and benzotriazolylalanine directly as substrates for the aminoacylation of 3′-[^{32}P]-$tRNA^{Phe}$ (Fig. 4). Benzofuranylalanine was found to be a substrate for aminoacylation by αA294G but not wild type PheRS, and benzotriazolylalanine was a substrate for neither enzyme. These results are consistent with the kinetic analyses and confirm that benzofuranylalanine, but not benzotriazolylalanine, can be attached to $tRNA^{Phe}$ by PheRS-αA294G. 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-αA294G 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-β-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-β-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-αA294G 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 α-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 benzofuranylalanine but showed no shtDHFR expression above background in the presence of benzotriazolylalanine (data now shown). Thus, benzofuranylalanine, but not benzotriazolylalanine, is a substrate for PheRS αT251/A294G.

Analysis of Benzofuran-containing shtDHFR—Because benzofuranylalanine (M_r 205) has a larger mass as compared with phenylalanine (M_r 165), incorporation of benzofuran into the reporter protein should be readily detectable as a significant mass increase of shtDHFR containing benzofuran. Mass spectrometry analysis of purified protein revealed molecular masses of 24,284 and 23,957 Da for shtDHFR expressed in the presence of benzofuranylalanine and phenylalanine, respectively (Fig. 6). This clearly demonstrates the incorporation of unnatural amino acid and corresponds to an average of ~8 benzofuranylalanine replacements in nine possible positions.

Conclusions—The data presented show that benzofuranylalanine, but not benzotriazolylalanine, is an efficient substrate for cellular protein synthesis as a result of its ability to be attached to $tRNA^{Phe}$ by PheRS-αA294G. These findings are of potential interest in the development of novel protein cross-linking methodologies given that benzofuranylalanine was found to be photoreactive at UVA wavelengths. The effective application of such methodologies is dependent on site-specific replacement of particular residues with benzofuranylalanine rather than global insertion in response to all phenylalanine codons as described here. Although a completely site-specific system may require extensive mutagenesis of both PheRS and a $tRNA^{Phe}$-derived suppressor species or perhaps another pair (4), existing heterologous codon-biased systems offer the potential to replace designated Phe residues with benzofuranylalanine in certain targets (21, 22).

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

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