# Supplementary Information

**Investigation of Amide Bond Formation during Dehydrophos Biosynthesis**

Emily C. Ulrich[1,2], Despina J. Bougioukou[2,†], and Wilfred A. van der Donk[1,2,3*]

[1]Department of Chemistry, [2]Carl R. Woese Institute for Genomic Biology, [3]Howard Hughes Medical Institute, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States, [†]Present address: E. I. du Pont de Nemours and Company, Wilmington, Delaware 19803, United States

*Correspondence to: E-mail: vddonk@illinois.edu

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**Supplementary Methods**

Reagents

*Molecular Biology:* Restriction enzymes (NdeI and DpnI), Phusion® polymerase, Gibson Assembly® master mix, and Taq DNA ligase were purchased from New England Biolabs, Inc. Fail Safe PCR buffers and polymerase and T5 exonuclease were purchased from Epicentre. DTT was purchased from Promega. dNTPs and $MgSO_4$ were purchased from EMB Millipore. Ampicillin (100 μg/mL) and chloramphenicol (12.5 μg/mL) were purchased from Fisher Scientific and Sigma Aldrich.

*tRNA Generation:* 10X NEBuffer™ 2, DNA polymerase I large Klenow fragment, rNTP mix, and thermostable inorganic pyrophosphatase (TIPP) were purchased from New England Biolabs, Inc. The dNTP mix was purchased from Novagen, Ambion® UltraPure™ BSA was purchased from Thermo Fisher Scientific. RNasin® RNase inhibitor and RNase-free DNase were purchased from Promega.

*Enzymology:* Nickel-nitrilotriacetic acid agarose was purchased from Qiagen. Isopropyl-β-D-galactopyranoside was purchased from IBI Scientific. Amicon ultracentrifugal filters were purchased from EMB Millipore. PD10 desalting columns were purchased from GE Healthcare. Chelex 100 resin, DNase, and (R)-(−)-1-aminoethylphosphonic acid (L-AlaP) were purchased from Sigma Aldrich. L-[$^{14}$C(U)]-amino acids and Ultima Gold cocktail were purchased from Perkin Elmer. Total tRNA from *E. coli* MRE 600 was purchased from Sigma Aldrich/Roche. The FemX(Wv)[1] and I-TASSER[2] structures were visualized using PyMOL.[3]

Cloning Procedures

New constructs for this study (pET-15b harboring the *E. coli* isoleucyl-tRNA synthetase and phenylalanyl-tRNA synthetase genes) were prepared using DH5α genomic DNA (Table S2) as template and following cloning procedures outlined previously.[4]

Upon sequencing pET-15b valRS, we found that it encodes the mutations I520V and Y688H compared to a NCBI reference sequence of *valRS* from DH5α (NZ_JRBB01000028.1). These may be the natural amino acids in the DH5α genomic DNA that we used.

Quikchange Mutagenesis

Reactions contained 1X Pfu Ultra buffer (Agilent Quikchange II Mutagenesis Kit), 0.6 ng/μL of pET-15b DhpH-C as template, 0.25 μM of each primer, 0.04 mM dNTPs (Novagen), 5% v/v DMSO, and 0.05 U/μL Pfu Ultra HF polymerase. For R290A, R296A, N325A, and E326A, the reactions contained 1X Buffer G (FailSafe kit), 0.6 ng/μL of pET-15b DhpH-C, 0.5 μM of each primer, and 0.04 U/μL of Phusion® HF polymerase (NEB). All of these reactions were run through the following protocol: 95 ºC for 1 min, 20 cycles of 95 ºC for 1 min, 60 ºC for 1.5 min, 72 ºC for 15 min, then 72 ºC for 5 min. For R233A, W284L, and R292A, the mutation was introduced using Gibson cloning.[5] For example, primers DhpHC_W284L_F and pET15b_NdeI_R were used to amplify around the vector, while DhpHC_W284L_R and NdeI-dhpH_C353 were used to amplify the gene with pET-15b DhpH-C as template. The reaction contained 1X of Buffer G (FailSafe kit), 0.5 μM of each primer, 0.9 ng/μL template, and 0.04 U/μL Phusion® polymerase. Each fragment was amplified using the following touch down PCR protocol: 98 ºC for 3 min, 30 cycles of 98 ºC for 10 s, 70 ºC for 30 s while dropping 0.5 ºC each round, 72 ºC 8 min (vector) or 2 min (gene), then 72 ºC for 10 min. All samples, regardless of amplification method, were treated with DpnI and purified using the QIAquick PCR purification

kit (Qiagen). For Gibson ligation, a 2:1 ratio of insert:vector was combined with assembly master mix from either NEB or prepared as previously described[4] (for W284L). Samples were incubated at 50 ºC for 1 h. For all samples, an aliquot (~3 μL) was used to transform 50 μL of electro-competent DH5α cells. Recovered cells were plated on LB/amp100 plates, which grew overnight at 37 ºC. Colonies were picked for plasmid preparation using the QIAprep Spin Miniprep Kit (Qiagen). Correct mutations were verified by sequencing either at ACGT, Inc. or the UIUC Core Sequencing Facility.

DhpH-C and aaRS Expression and Purification

All proteins were expressed and purified as previously described.[4]

In vitro Transcription of $tRNA^{Leu}$

Sequences for *E. coli* $tRNA^{Leu}$ (UAG) and (GAG) were obtained from the EcoCyc database.[6] dsDNA templates were prepared based on a previously described method.[7] A 500 μL reaction for template generation contained: 1X Buffer 2 (New England Biolabs, Inc.), 4 μM of each primer, 40 μM of dNTPs, and 0.06 U/μL DNA polymerase I (large Klenow fragment). The reaction was incubated at 25 ºC for 15 min before the addition of EDTA (10 mM final concentration) and inactivation by heating at 75 ºC for 20 min. The DNA was precipitated in EtOH overnight at -20 ºC. The resulting pellet was washed three times with ice cold 75% EtOH and air-dried before resuspension in nuclease-free water. The entire template yield was used in either a 1000 or 1500 μL *in vitro* transcription reaction based on a previously described method.[8] The reaction contained 1X transcription buffer (100 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, and 0.1 mg/mL RNase-free BSA), 10 mM DTT, 26 mM $MgCl_2$, 7.5 mM of each NTP, 0.8 U/μL RNasin® RNase inhibitor, 0.01 U/μL TIPP, ~30 ng/μL template DNA, and 140 ng/μL T7 RNA polymerase. The reaction was incubated overnight at 37 ºC, then 5 U of RNase-free DNase was added per 500 μL transcription reaction, and incubation continued for 1 h at 37 ºC. The reaction was desalted by filtration using a 10 kDa molecular weight cut-off centrifugal filter (Millipore; 4 wash cycles with 50 mM HEPES pH 7.5). The tRNA was purified using an acidic phenol / 24:1 chloroform:isoamyl alcohol extraction described previously, ending with precipitation in EtOH and resuspension in 2 mM NaOAc pH 5.2.[9] The tRNA was precipitated in EtOH overnight at -20 ºC. The resulting pellet was washed with ice cold 75% EtOH and air-dried before resuspension in 2 mM NaOAc pH 5.2. tRNA concentration was determined using a Qubit® 2.0 Fluorometer and the RNA Broad-Range assay kit (Thermo Fisher Scientific).

Aminoacylation Assay for in vitro Transcribed and Total *E. coli* tRNAs

The reactions contained 37.5 mM HEPES pH 7.5, 112.5 mM KCl, 11.2 mM $MgCl_2$, 5 mM ATP, 0.01 U/μL TIPP, 8 μM LeuRS, 50 μM L-[$^{14}$C(U)]-Leucine (328 mCi/mmol), and 16 μM $tRNA^{Leu}$. Each solution was incubated at room temperature (to replicate conditions for DhpH-C activity assays) for 15 min (1 h for LeuRS*m assays) before quenching and preparation for analysis adapted from previously described protocols.[4, 10-12] Briefly, an aliquot of 5 μL was spotted onto 3MM Whatman filter paper squares presoaked in 5% trichloroacetic acid (TCA). The spots were allowed to dry for 5 min before the filter paper squares were washed 3 times in cold 5% TCA and once in cold 75% EtOH for 5 min each. The squares were air-dried and then analyzed by liquid scintillation counting in Ultima Gold scintillation cocktail using a Tri-Carb 2910TR. Experiments were performed in triplicate. Results were plotted using Igor Pro version 6.32A.

Aminoacylation assays with total *E. coli* tRNA were performed as described above, except that 3 mg/mL tRNA was used initially before adjusting the amount of tRNA to achieve equivalent aminoacylation levels for each aaRS (see amounts described for the activity assay). In these assays, 8 μM of each aaRS was used along with the corresponding amino acid: L-[$^{14}$C(U)]-isoleucine (308 mCi/mmol), L-[$^{14}$C(U)]-valine (271 mCi/mmol), and L-[$^{14}$C(U)]-phenylalanine (477 mCi/mmol).

Liquid Chromatography-Mass Spectrometry (LCMS) Analysis of aaRS Endpoint Assays

To verify that the masses of the proposed dipeptides are present in the samples with various amino acids, samples of DhpH-C with IleRS, ValRS, PheRS, and LeuRS*m that had been quenched after 3 h were analyzed by LCMS. A sample with no DhpH-C and the Leu/LeuRS aa-tRNA generation system was used as background. Samples were analyzed by using the Q-Exactive MS system (Thermo. Bremen, Germany) in the Metabolomics Laboratory of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Xcalibur 4.1.31.9 was used for data acquisition and analysis. The Dionex Ultimate 3000 series HPLC system (Thermo, Germering, Germany) used includes a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex RP Fusion column (4.6 x 150 mm, 4 μm) with mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The flow rate was 1 mL/min. The linear gradient was as follows: 0 min, 100% A; 5-15 min, 5% A; 16-22 min, 100% A. The autosampler was set to 15°C. The injection volume was 10 μL. Mass spectra were acquired with electrospray ionization under both positive and negative mode: sheath gas flow rate, 65; aux gas flow rate: 20; sweep gas flow rate, 4; spray voltage, 4.0 kV; capillary temp, 300 °C; Aux gas heater temp, 500 °C. The resolution was set to 70,000 with the scan range of m/z 50 - 600. The AGC target was 1E6 with a maximum injection time of 200 ms.

Table S1. Primer Sequences

All primers were purchased from Integrated DNA Technologies, Inc.

| Name | Sequence (mutation is in bold) |
|---|---|
| DhpHC_R233A_F | gccgccagcgccacc**gcc**accggctcgcccaag |
| DhpHC_R233A_R | cttgggcgagccggt**ggc**ggtggcgctggcggc |
| DhpHC_W284L_F | caagcgcctgcaggca**ctc**gccggagggtatgtg |
| DhpHC_W284L_R | cacataccctccggc**gag**tgcctgcaggcgcttg |
| DhpHC_R290A_F | gccggagggtatgtg**gcc**ggccgcgccgacctg |
| DhpHC_R290A_R | caggtcggcgcggcc**ggc**cacataccctccggc |
| DhpHC_R292A_F | gggtatgtgcgcggc**gcc**gccgacctgcggttc |
| DhpHC_R292A_R | gaaccgcaggtcggc**ggc**gccgcgcacataccc |
| DhpHC_R296A_F | ggccgcgccgacctg**gcg**ttcagcccgtactac |
| DhpHC_R296A_R | gtagtacgggctgaa**cgc**caggtcggcgcggcc |
| DhpHC_N325A_F | gtgcggccggctc**gcc**gaatccttcaaggagaag |
| DhpHC_N325A_R | cttctccttgaaggattc**ggc**gagccggccgcac |
| DhpHC_E326A_F | gtgcggccggctcaac**gca**tccttcaaggagaag |
| DhpHC_E326A_R | cttctccttgaagga**tgc**gttgagccggccgcac |
| DhpHC_F328L_F | ccggctcaacgaatcc**ctc**aaggagaagatgctc |

| DhpHC_F328L_R | gagcatcttctcctt**gag**ggattcgttgagccgg |
|---|---|
| DhpHC_K329M_F | ccggctcaacgaatccttc**atg**gagaagatgctcctc |
| DhpHC_K329M_R | gaggagcatcttctc**cat**gaaggattcgttgagccgg |
| DhpHC_K329R_F | ccggctcaacgaatccttc**agg**gagaagatgctcctc |
| DhpHC_K329R_R | gaggagcatcttctc**cct**gaaggattcgttgagccgg |
| pET15b_XhoI_F | ctcgaggatccggctgctaacaaagcccgaaagg |
| pET15b_NdeI_R | catatggctgccgcgcggcaccaggccgctg |
| NdeI-dhpH_C353 | gcagcggcctggtgccgcgcggcagccatatgctggacggttcggccggcggccccgc |
| Ecoli_IleRS_F | gcagcggcctggtgccgcgcggcagccatatgagtgactataaatcaaccctgaatttgccg |
| Ecoli_IleRS_R | cctttcgggctttgttagcagccggatcctcgagtcaggcaaacttacgtttttcaccgtcaccggcg |
| Ecoli_PheRS_F | gcagcggcctggtgccgcgcggcagccatatgtcacatctcgcagaactggttgccagtgcg |
| Ecoli_PheRS_R | cctttcgggctttgttagcagccggatcctcgagtcaatccctcaatgatgcctggaatcgctc |
| $tRNA^{Leu}$ TAG F | AATTCCTGCAGTAATACGACTCACTATAGCGGGAGTGGCGAA ATTGGTAGACGCACCA |
| $tRNA^{Leu}$ TAG R* | mUmGGTGCGGGAGGCGAGACTTGAACTCGCACACCTTGCGGCG CCAGAACCTAAATCTGGTGCGTCT |
| $tRNA^{Leu}$ GAG F | AATTCCTGCAGTAATACGACTCACTATAGCCGAGGTGGTGGAA TTGGTAGACACGCTA |
| $tRNA^{Leu}$ GAG R* | mUmGGTACCGAGGACGGGACTTGAACCCGTAAGCCCTATT GGGCACTACCACCTCAAGGTAGCGTGTCT |
| $tRNA^{Leu}$ GAG (GC) R* | mUmGGT**G**CCGAGGACGGGACTTGAACCCGTAAGCCCTATTGG GCACTACCACCTCAAGGTAGCGTGTCT |
| $tRNA^{Leu}$ GAG (AU/AC) F | AATTCCTGCAGTAATACGACTCACTATAACCGAGGTGGTGG AATTGGTAGACACGCTA |

* mU, mG: 2’-*O*-methylated nucleotide

Table S2. Strains and Plasmids

| Name | Features | Source |
|---|---|---|
| *E. coli* DH5α λpir | sup E44, ΔlacU169 (ϕlacZΔM15), recA1,endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen. | ([13]) |
| *E. coli* Rosetta 2 (DE3)pLysS | $CAM^R$; provides seven rare, in *E. coli*, tRNAs for the codons CGG, AUA, AGG, AGA, CUA, CCC, and GGA in the same plasmid that habors the T7 lysozyme | Novagen |
| pET-15b | $AMP^R$; N-terminal 6xHis-tag® of protein, includes a thrombin recognition sequence | Novagen |
| pET-15b dhpH-C | $AMP^R$ (NdeI/XhoI) | ([4]) |
| pET-15b dhpH-C R233A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C W284L | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C R290A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C R292A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C R296A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C N325A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C E326A | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C F328L | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C K329M | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b dhpH-C K329R | $AMP^R$ (NdeI/XhoI) | This study |
| pET-14b leuRS | $AMP^R$ | Gift from the Martinis lab (UIUC) ([4, 14]) |
| pET-15b leuRS Y330A/D342A/D345A | $AMP^R$ | Gift from the Martinis lab (UIUC) ([15]) |
| pET-15b ileRS | $AMP^R$ (NdeI/XhoI) | This study |
| pET-15b valRS | $AMP^R$ (NdeI/XhoI) | ([16]) |
| pET-15b pheRS | $AMP^R$ (NdeI/XhoI) | This study |

## Supplementary Figures

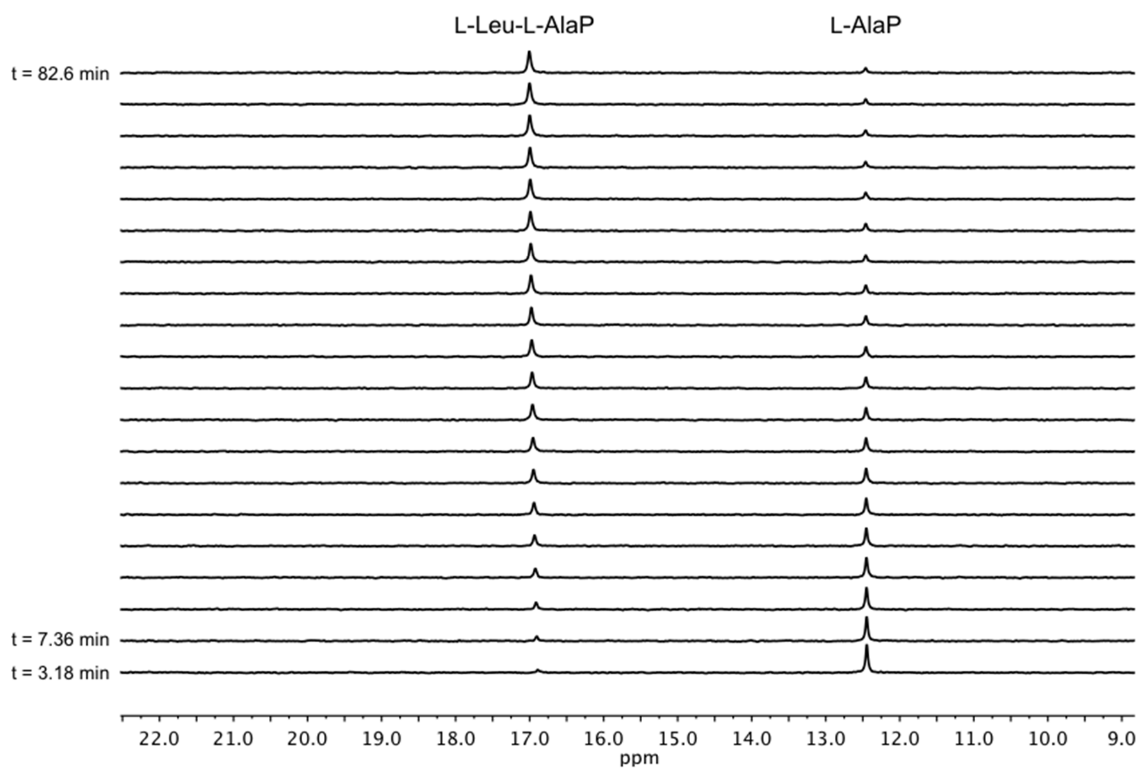


**Figure S1. Example $^{31}$P NMR spectra collected to follow the DhpH-C reaction over time.** The phosphonate spectral region of an example series of array data depicting product formation over roughly 80 min for the reaction with DhpH-C E326A. Spectra were collected roughly every 4 min.

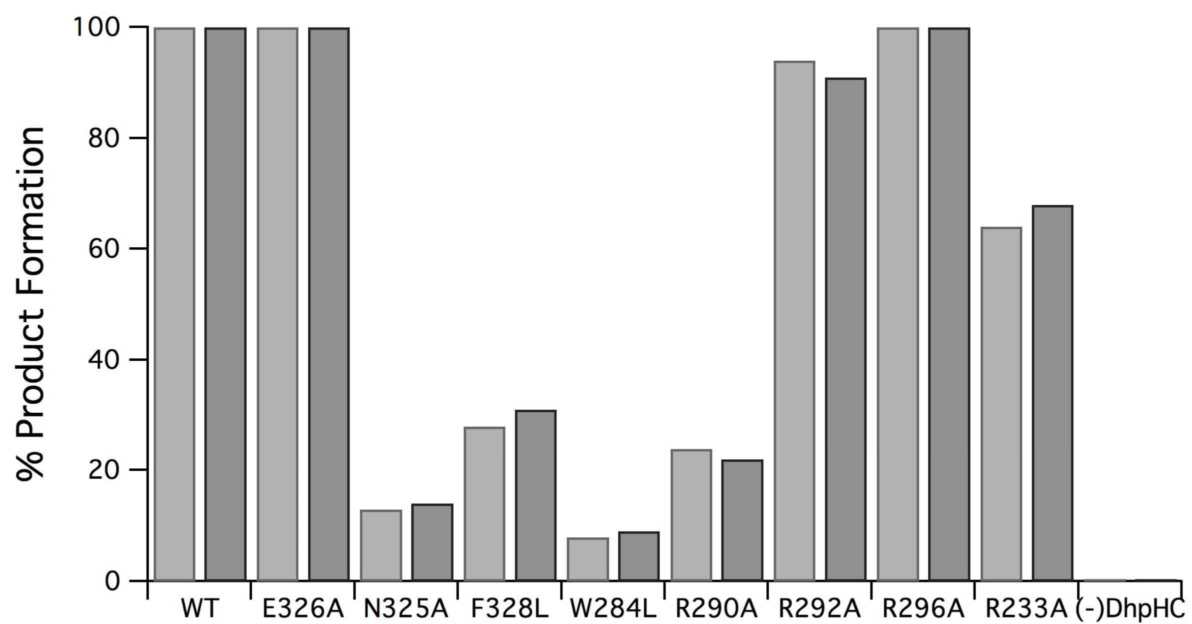


**Figure S2. Product formation after 3 h for DhpH-C variants.** Experiments were performed in duplicate.

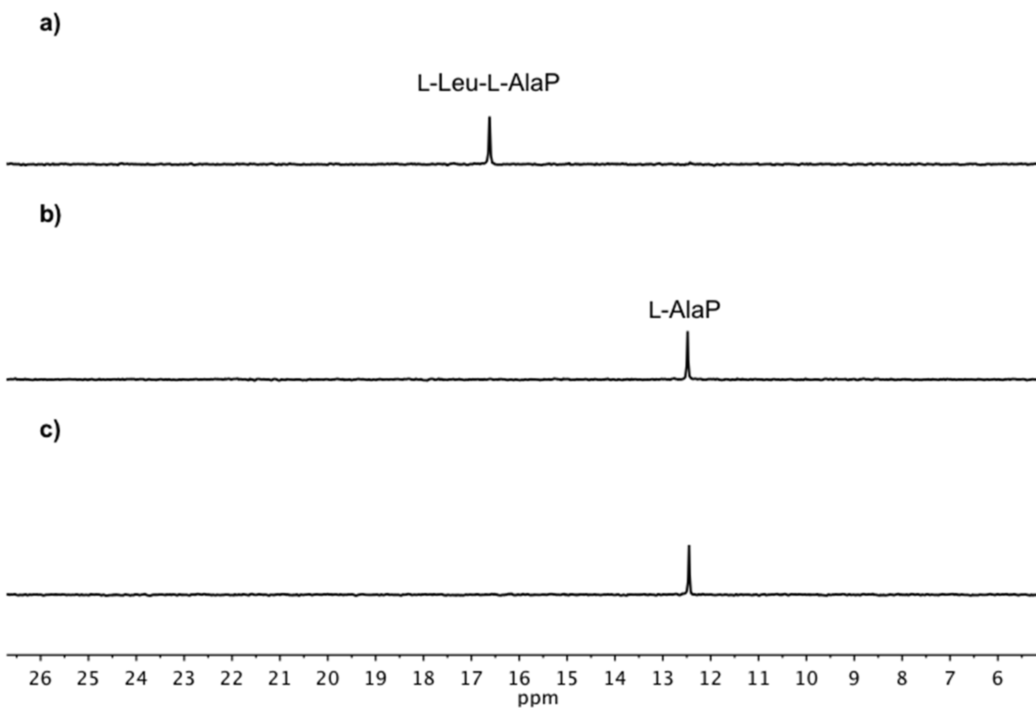


**Figure S3. No product is observed with DhpH-C K329M/R.** $^{31}P$ NMR spectra after a 12 h reaction with a) DhpH-C WT, b) DhpH-C K329R, or c) DhpH-C K329M. Reactions were performed in duplicate with 30 μM enzyme.

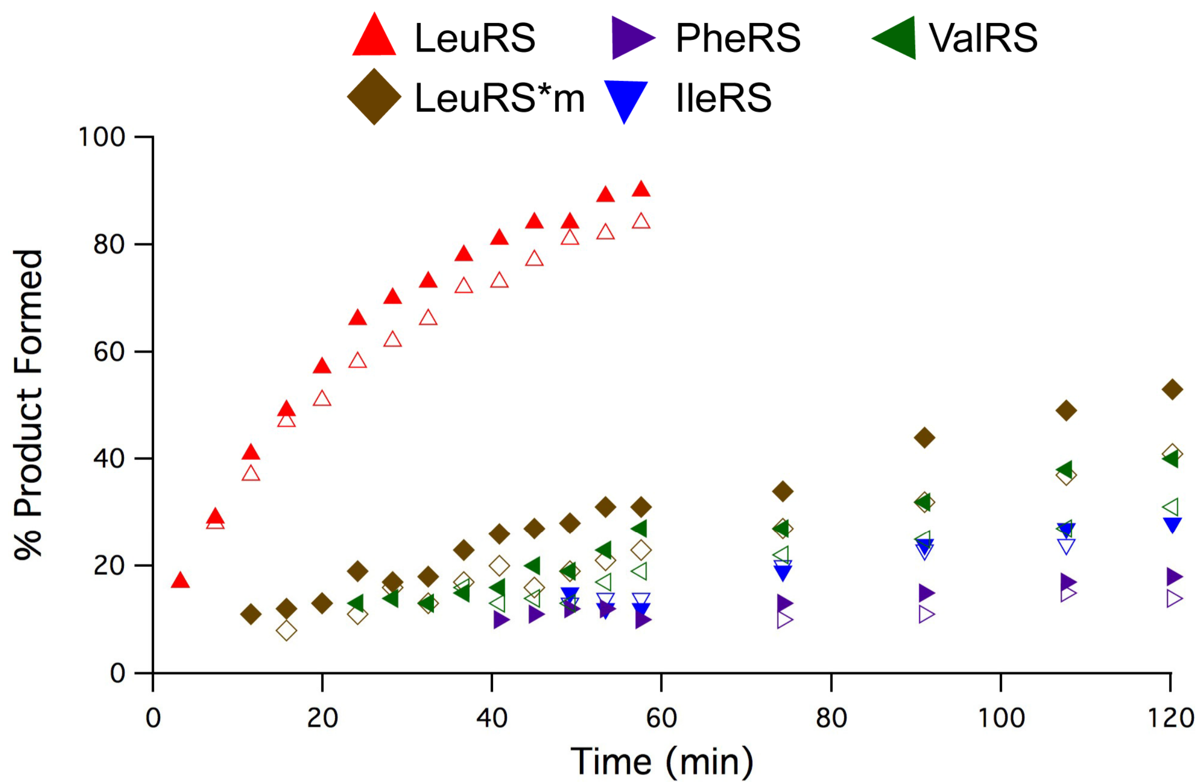


**Figure S4. Reaction rate does not increase when doubling the aaRS concentration.** Reactions were set up with 3 mg/mL total tRNA and 35 µM DhpH-C WT (10 µM for the LeuRS sample). Spectra were collected every 4 min for 2 h. Reactions contained 8 µM synthetase (filled markers) or 16 µM synthetase (open markers).

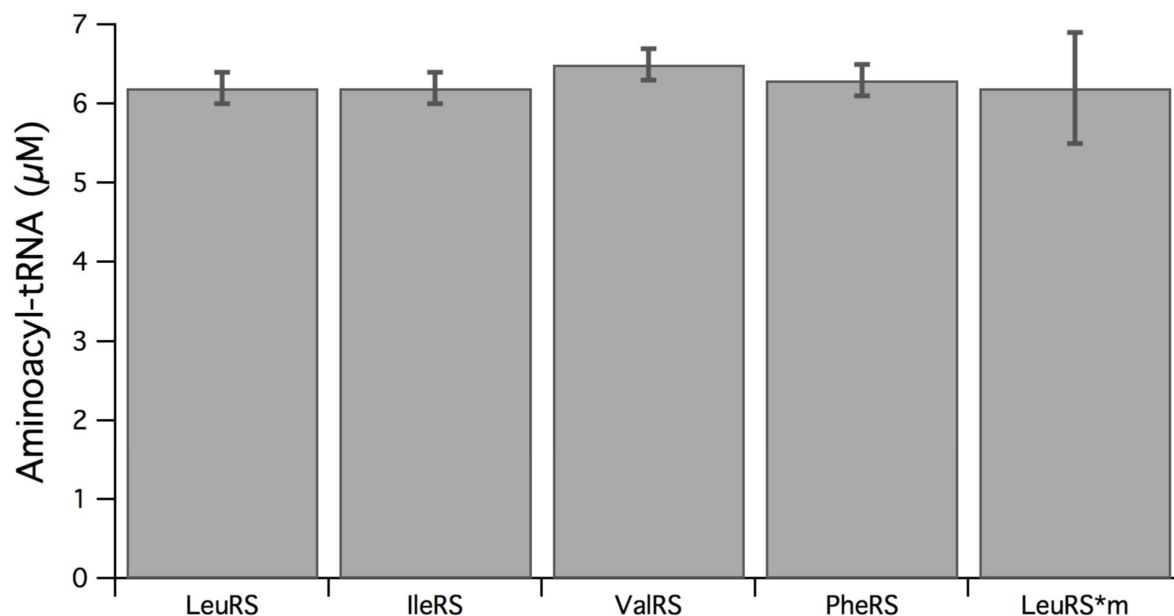


**Figure S5. Determination of conditions to achieve equivalent aminoacylation levels for each synthetase.** Aminoacylation experiments were performed with different amounts of total *E. coli* tRNA to achieve comparable amounts of aminoacylated tRNA. The final conditions identified are shown. Amount of total *E. coli* tRNA used: 3 mg/mL (LeuRS), 5.1 mg/mL (IleRS), 3.9 mg/mL (ValRS), 9.6 mg/mL (PheRS), and 5.4 mg/mL (LeuRS*m). For experimental details, see the section on the aminoacylation assay for total *E.coli* tRNAs. The bar graph represents the average of three replicates, with error bars indicating the standard deviation.

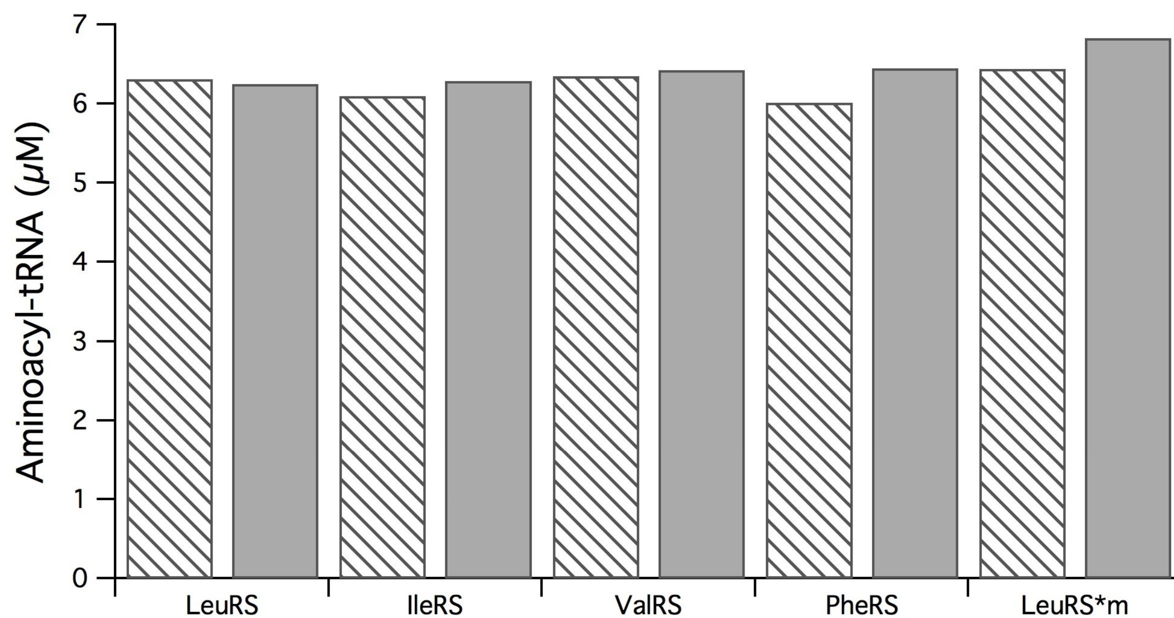


**Figure S6. The aminoacylation reaction with various synthetases has gone to completion.** Aminoacylation reaction aliquots were quenched after 15 min (striped) and 30 min (solid), or 1 h (striped) and 2 h (solid) for LeuRS*m to determine if the reaction had gone to completion.

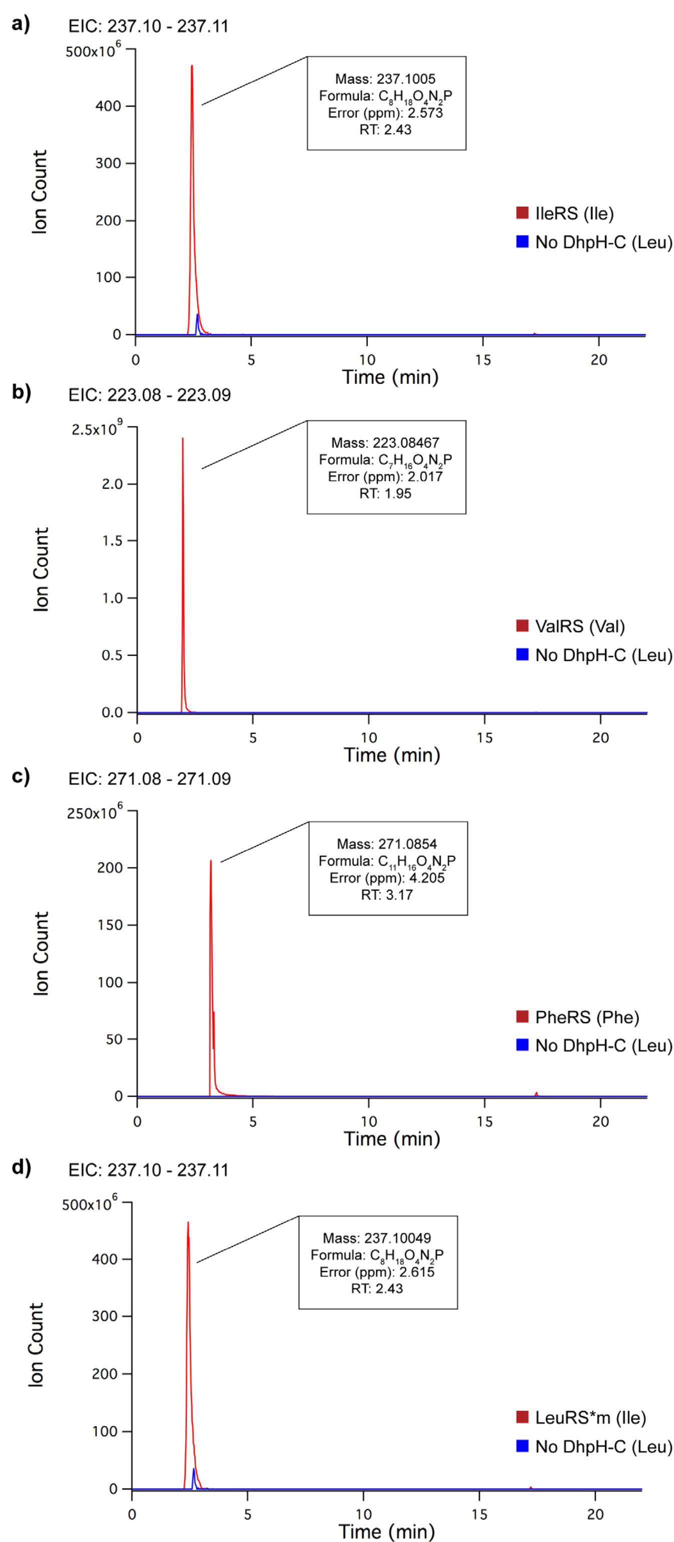


**Figure S7. Analysis of dipeptide product formation by LCMS.** The mass of the predicted dipeptide products with IleRS, ValRS, PheRS, and LeuRS*m was confirmed for samples that had been incubated for 3 h. Each extracted ion chromatogram of the indicated dipeptide is compared to a sample with Leu/LeuRS and no DhpH-C.

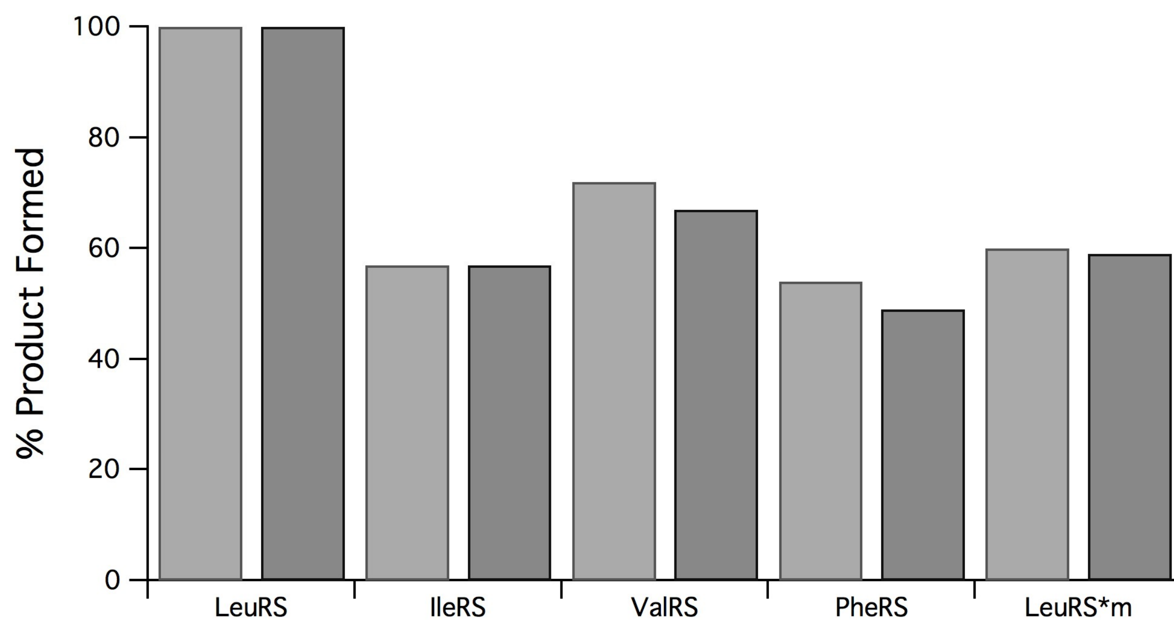


**Figure S8. Product formation after 3 h with different amino acid/aaRS pairs.** Experiments were performed in duplicate with total *E. coli* tRNA, 35 μM DhpH-C, and 8 μM aaRS.

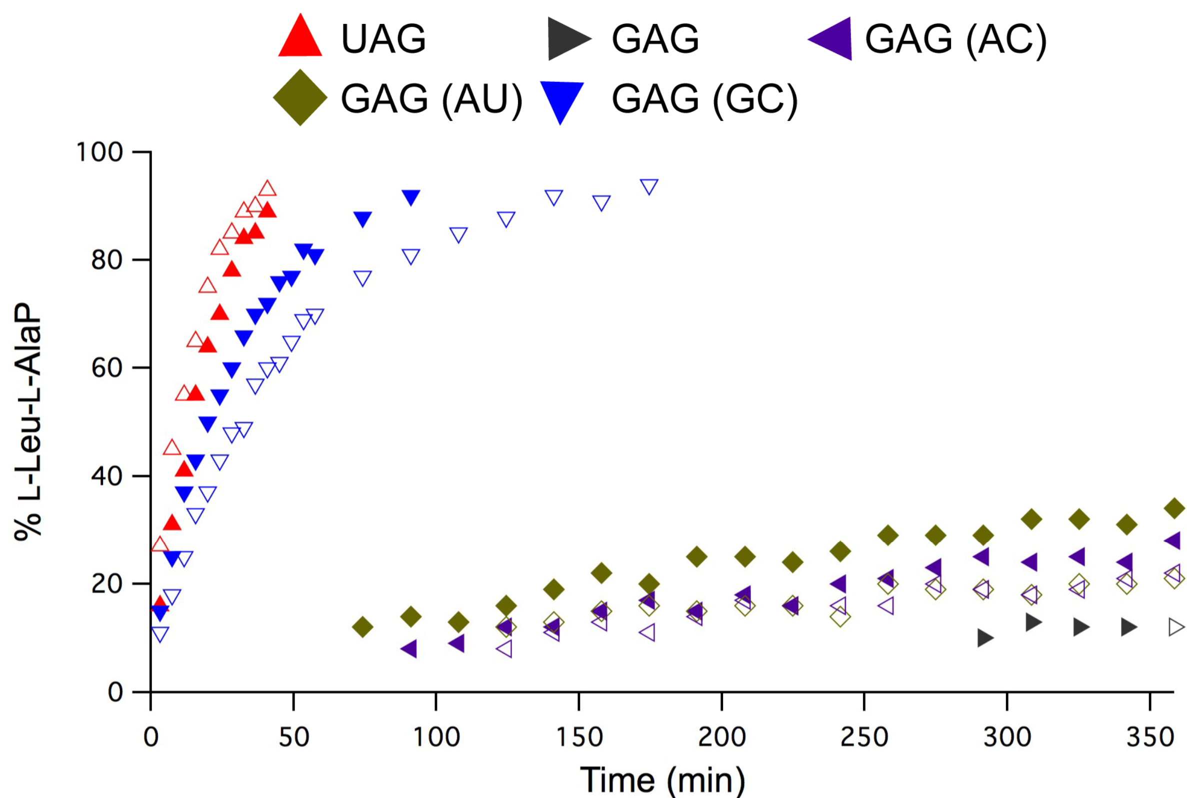


**Figure S9. Reaction rate with various tRNA$^{Leu}$ variants does not increase when the LeuRS concentration is doubled.** Reactions were set up with 16 µM tRNA$^{Leu}$ variants (8 µM for GAG (AU)) and 10 µM DhpH-C WT. Reactions contained 8 µM LeuRS (filled markers) or 16 µM LeuRS (open markers).

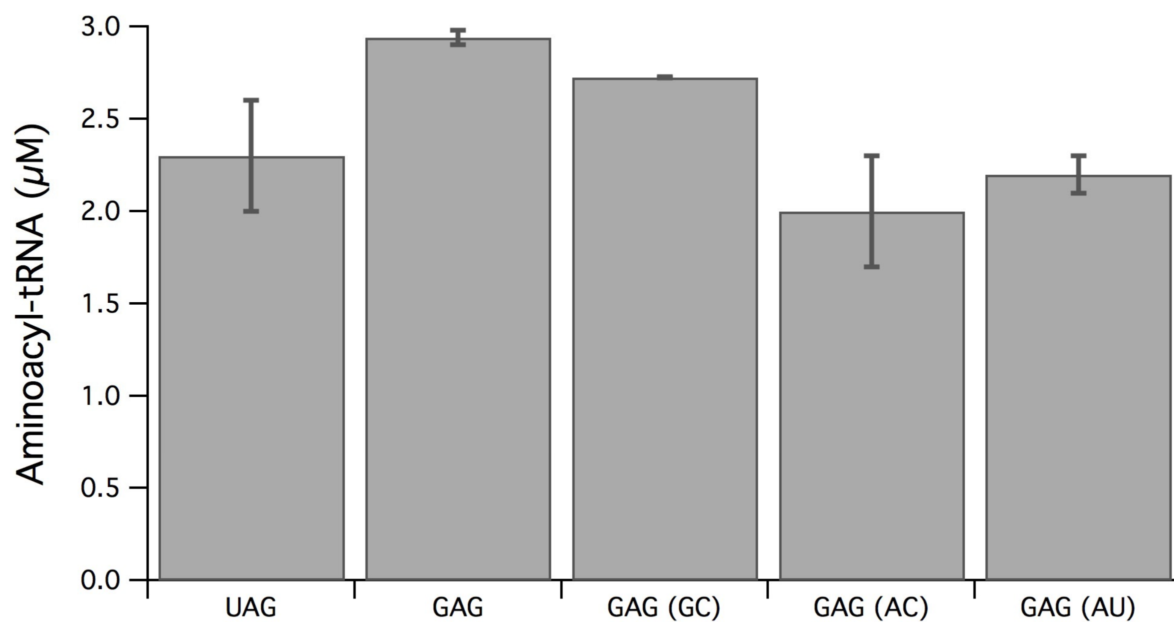


**Figure S10. Determination of conditions to achieve equivalent aminoacylation levels with each tRNA$^{Leu}$ variant.** Aminoacylation experiments were performed with 16 μM tRNA$^{Leu}$, except 8 μM was used for GAG (AU). The bar graph represents the average of three replicates, with error bars indicating the standard deviation.

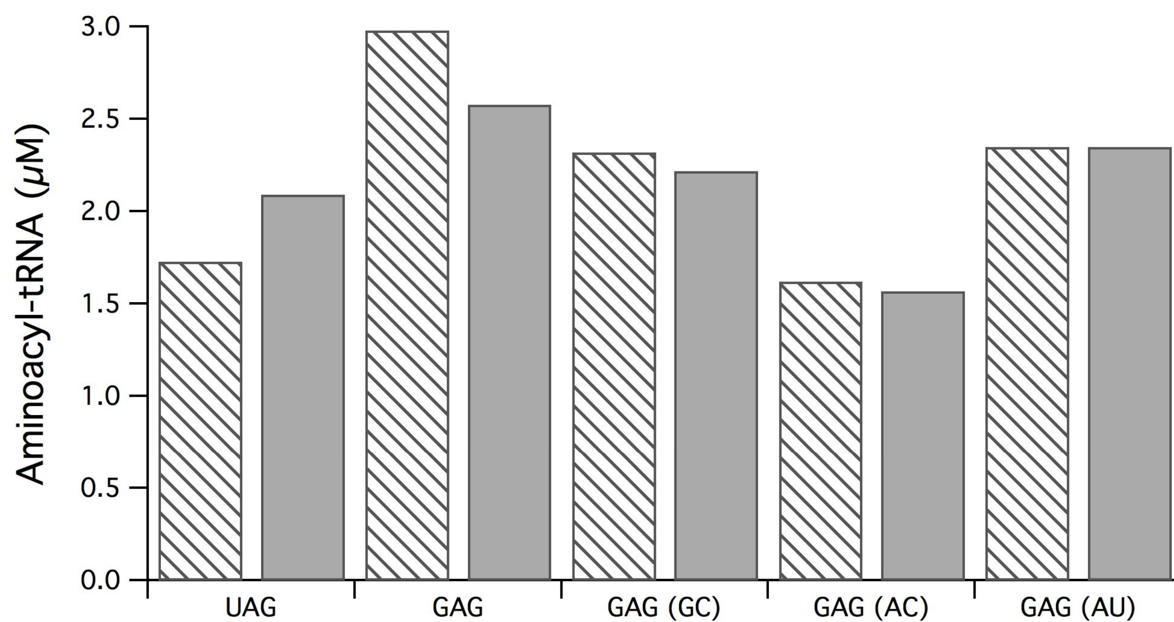


**Figure S11. The aminoacylation reaction with tRNA$^{Leu}$ variants has gone to completion.** Aminoacylation experiments were performed with 16 μM tRNA$^{Leu}$, except 8 μM was used for GAG (AU). Reaction aliquots were quenched after 15 min (striped) and 30 min (solid) to determine if the reaction had gone to completion.

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