Supporting Information

Ribosomal incorporation of backbone modified amino acids via an editing-deficient aminoacyl-tRNA synthetase

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AARS Charging Assay MALDI-TOF *m/z* $[M+H]^+$		
	Calculated	Observed
β-Azidohomoalanine	882.2893	882.1639
β-Cyano-L-Alanine	852.2675	852.1853
Norleucine	869.3192	869.2125
Crotyl Glycine	867.3036	867.1818
L-*allo* -Threonine	857.2828	857.1826
2-Aminobutyric Acid	841.2879	841.2039
β-1	881.2970	881.3192
β-2	881.2970	881.3113
α-Methyl Serine	857.2828	857.2565
α-Methyl Alanine	841.2879	841.2840
Valine	855.3036	855.2878

In vitro Translation MALDI-TOF *m/z* $[M+H]^+$		
	Calculated	Observed
β-Azidohomoalanine	1257.5020	1257.2924
β-Cyano-L-Alanine	1227.4802	1227.4861
Norleucine	1245.5319	1245.4066
Crotyl Glycine	1242.5163	1242.5664
L-*allo* -Threonine	1232.4955	1232.5212
2-Aminobutyric Acid	1216.5006	1216.1567
β-1	1256.5319	1256.5753
β-2	1256.5319	1256.4271
α-Methyl Serine	1232.4955	1232.4895
α-Methyl Alanine	1216.5006	1216.2604
Valine	1230.5163	1230.6245

Table S1. Calculated and Observed MALDI-TOF *m/z*. $[M+H]^+$ for ncAAs and Valine (last entry) for AARS Charging Assay (left table) and *in vitro* translations (right table).

Amino Acid	Peptide Yield (pmoles)
Val	7.96 ± 0.27
β-1	5.34 ± 0.01
β-2	5.10 ± 0.31
αSer	5.04 ± 0.30
αAla	4.18 ± 0.29
αSer Dual	6.01 ± 0.18
αAla Dual	6.26 ± 0.22
Aha	6.12 ± 0.11
CrG	6.11 ± 0.10
Nle	5.34 ± 0.23
Abu	5.30 ± 0.20
BCA	5.16 ± 0.26
A-Thr	4.65 ± 0.23
-tRNA	0.63 ± 0.01
-ncAA	0.60 ± 0.01

Table S2. Incorporation of ncAAs. Translational yields of peptides containing ncAAs as measured by ^{35}S-Methionine incorporation. "-tRNA" corresponds to an assay with no precharged tRNA added to translation. "-ncAA" corresponds to an assay containing added tRNA that was pre-charged in the absence of the ncAA. The yield of the peptides was calculated using the radioactive counts of the capture peptide after subtraction of a background translation experiment lacking mRNA.

tRNA^{Val} (GUA) Sequence: GGGUGAUUAGCUCAGCUGGGAGAGCACCUCCCUUACACGGAGGGGGUCGGCGGUUCGAUCCCGUCAUCACCCACCA					
Primer Extention		1st PCR		2nd PCR	
FWD	REV	FWD	REV	FWD	REV
V-1	V-2	V-4	V-3	V-4	V-5
V-1	GTAATACGACTCACTATAGGGTGATTAGCTCAGCTGGGAGAGCACCTCCC				
V-2	GAACCGCCGACCCCCTCCATGTAAGGGAGGTGCTCTCCCAGCT				
V-3	TGGTGGGTGATGACGGGATCGAACCGCCGACCCCCTCC				
V-4	GGCGTAATACGACTCACTATAG				
V-5	TmGGTGGGTGATGACGGGATC				

Table S3. tRNA^{Val} construction. PCR Primers used for tRNA^{Val} DNA template construction. V-5 contains a 2' O-methylated guanine base ("mG") to reduce non-templated nucleotide addition at the 3' terminus of the DNA template to be used for tRNA transcription.[1]

Name	Sequence
tRNA Val GUA	TGG TGG GTG ATG ACG GGA TCG AAC CGC CGA CCC CCT CCT TGT AAG GGA GGT GCT CTC CCA GCT GAG CTA ATC ACC CTA TAG TGA GTC GTA TTA
T7 Forward	TAATACGACTCACTATAGGG

Table S4. tRNA^{Val} transcription primers for the AARS charging assay.

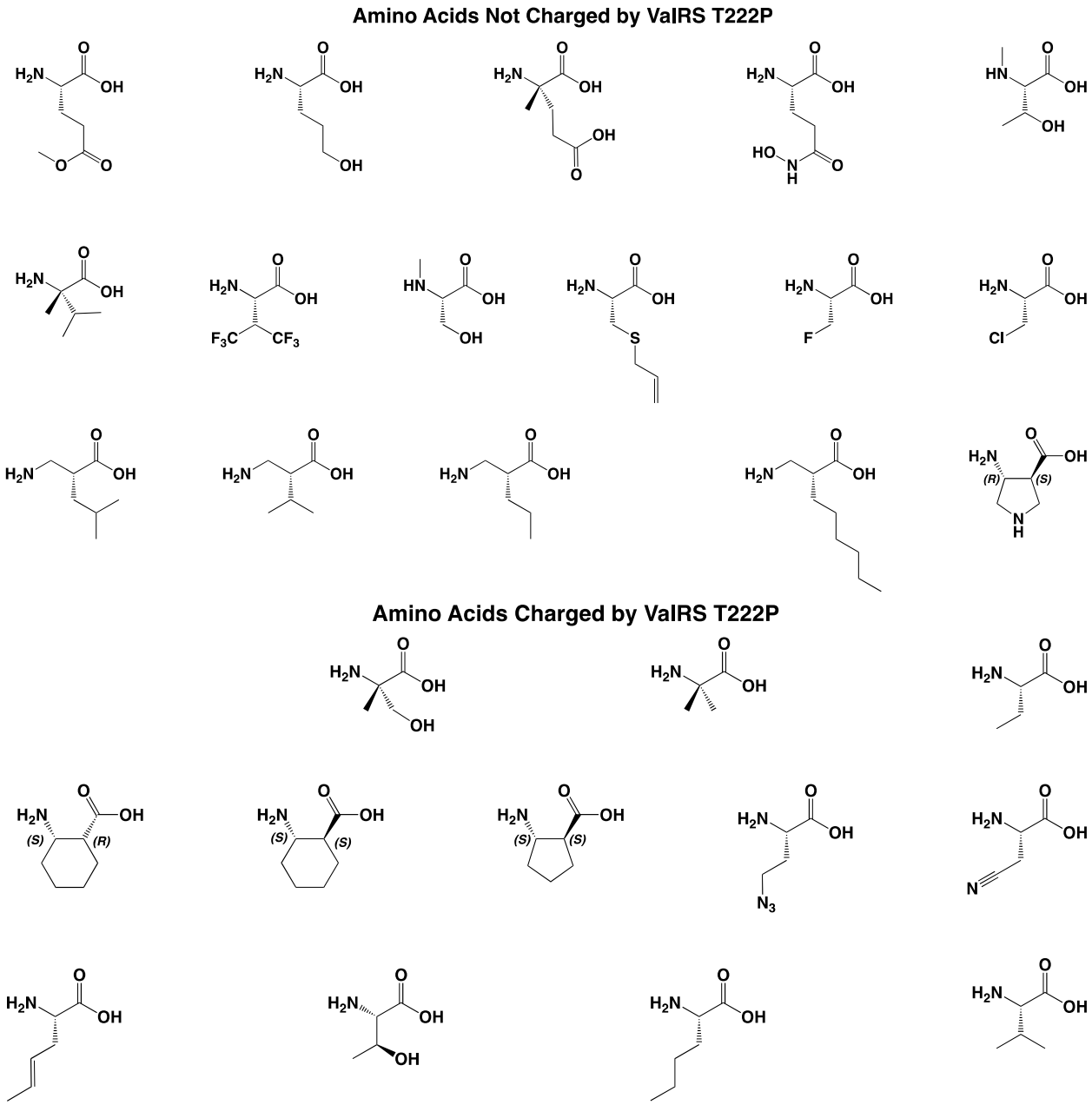

Figure S1. ncAAs Test for Aminoacylation by ValRS T222P.

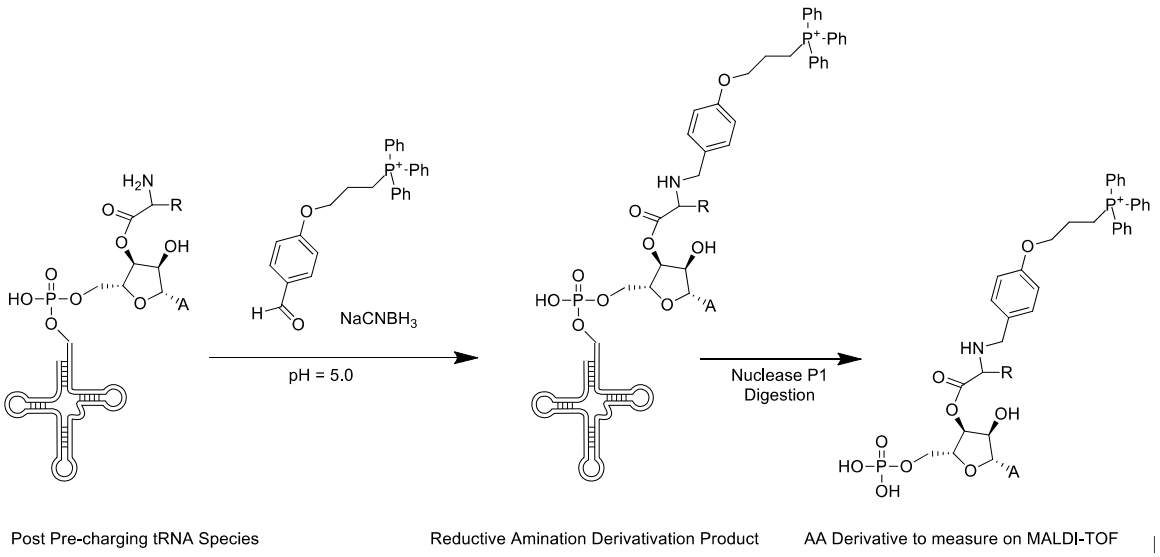

Figure S2. Overview of AARS Charging Assay. The aminoacylated tRNA species is subjected to reductive amination at the free amino group of the amino acid with a triphenylphosonium benzaldehyde reagent. The resulting AA-tRNA-triphenylphosphonium species is digested with Nuclease P1 to yield an AA-AMP species easily detectable by MALDI-TOF.

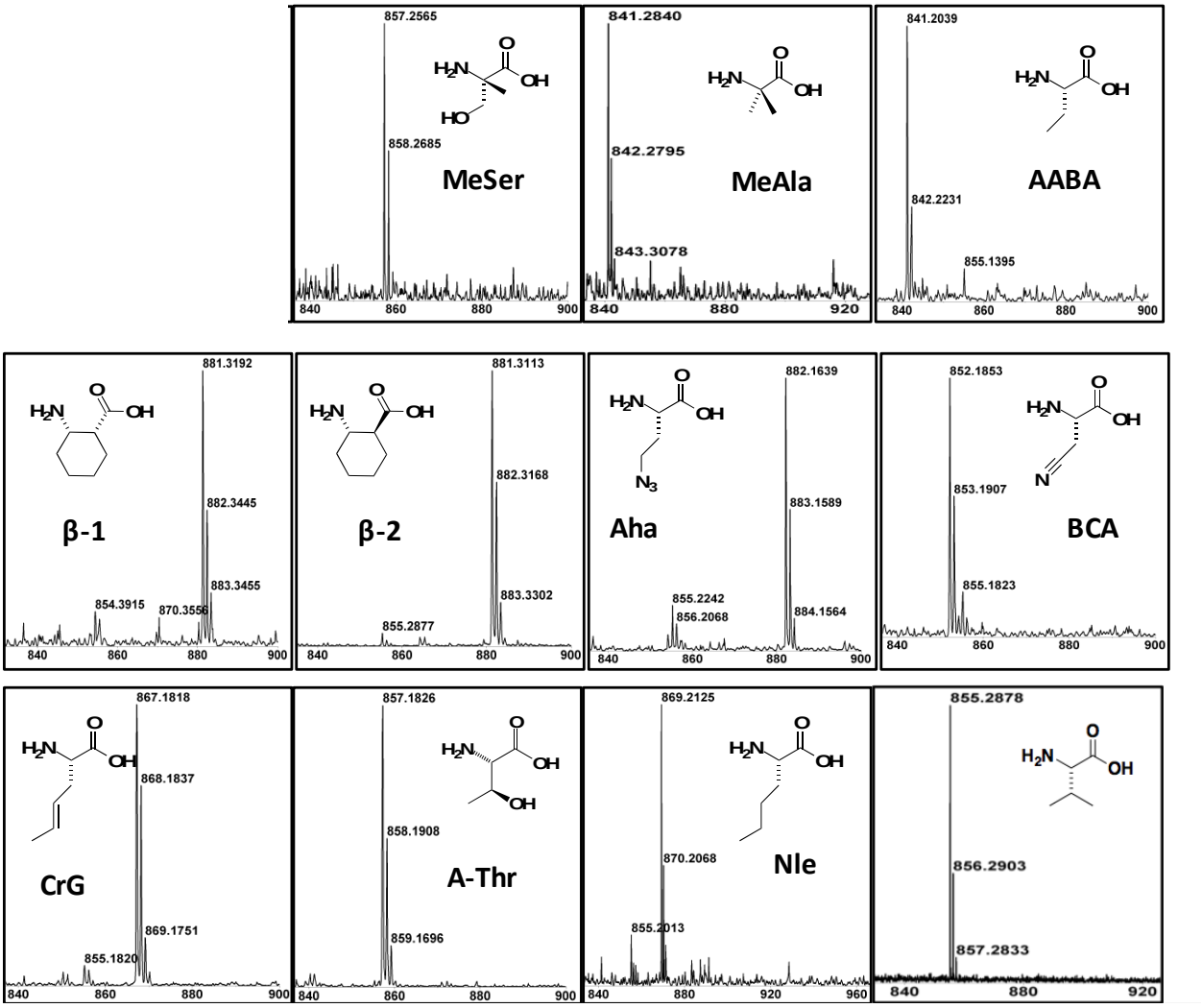

Figure S3. MALDI-TOF MS AARS assay showing isotopic resolution. Charging Assay of each ncAA charged with ValRS T222P. The major peak in each assay corresponds to the derivatized amino acid. In certain cases small peaks from charging of valine are present (calc. 855.3031 Da). See Table S1 for expected masses.

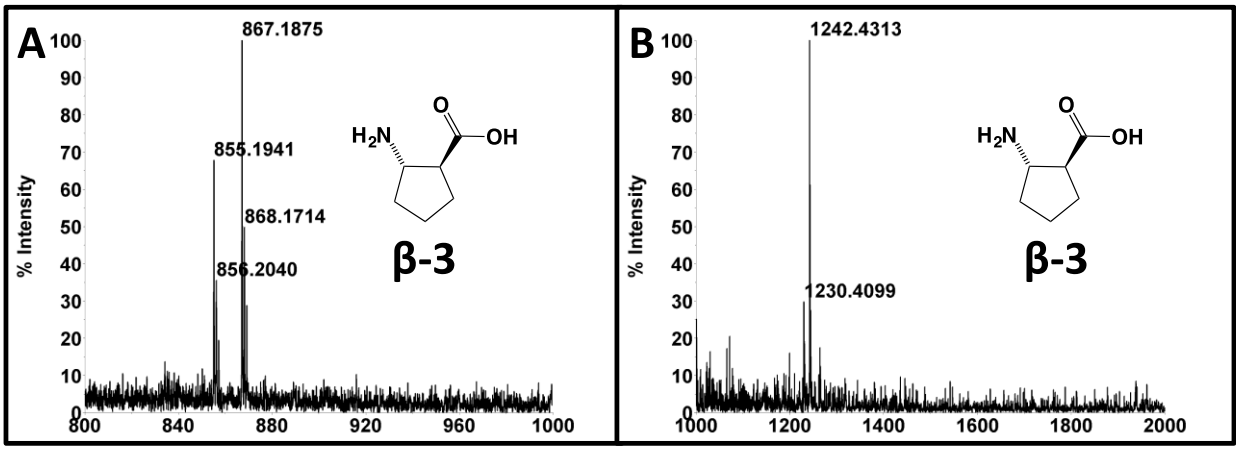

Figure S4. Charging and Translation of β-3. (A) Mass spectrum of charging assay where VRS T222P is incubated with β-3. Expected *m/z*: 867.3036. Observed *m/z*: 867.18. Peak at *m/z* = 855.19 corresponds to valine contamination in sample. (B) Translation using precharged tRNA from (A) and mRNA template from Figure 2. Expected *m/z*: 1242.57. Observed *m/z*: 1242.43. The peak at *m/z* = 1230.41 corresponds to a valine containing peptide. The yield from the translation reaction was 3.1 ± 0.2 pmols.

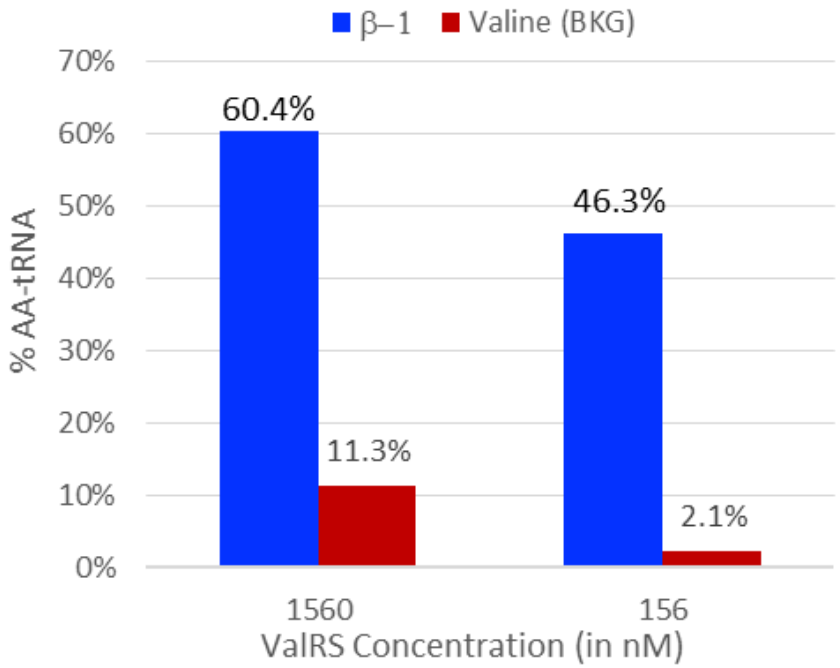

Figure S5. Minimizing Background Charging of Valine. A ten-fold dilution of ValRS T222P was used in the ^{32}P charging assay charging assay to minimize background valine charging while maintaining activity for β-1. Blue bars show the % AA-tRNA formed after 1 h reaction in the presence of 10 mM β-1. Red bars represent identical assays in the absence of β-1, and, therefore, represent background valine incorporation. Upon 10-fold dilution of the enzyme, the background valine contamination was reduced significantly, yet the ability to charge β-1 was maintained, indicating that the valine source was the enzyme stock itself. Bars are indicative of singlet analysis.

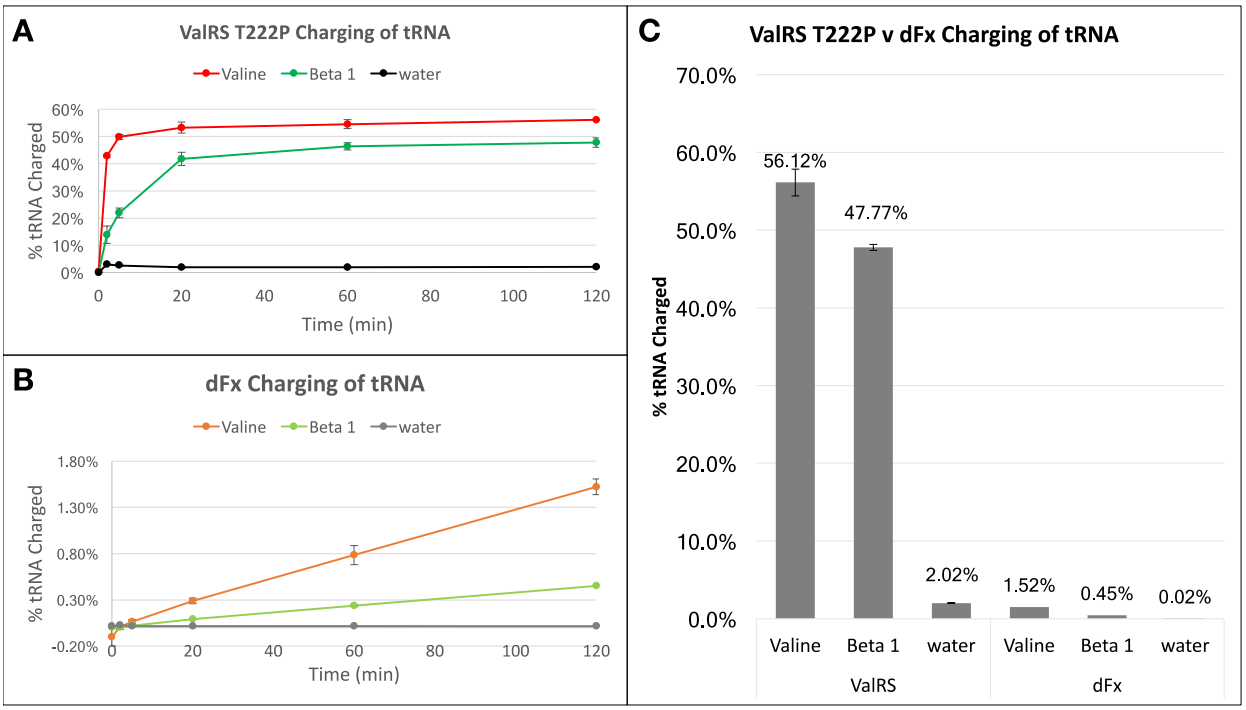

Figure S6. ^{32}P-Charging assay for tRNA^{Val}. (A) Time course of charging of tRNA^{Val} with ValRS Valine (red), β-1 (green). Black shows background charging with no AA added. (B) Time course of charging of tRNA^{Val} with dFx and valine (red) and β-1 (green). Grey shows background charging with no activated AA added. (C). Total tRNA^{Val} charging percentage after 2 h incubation under the standard conditions.

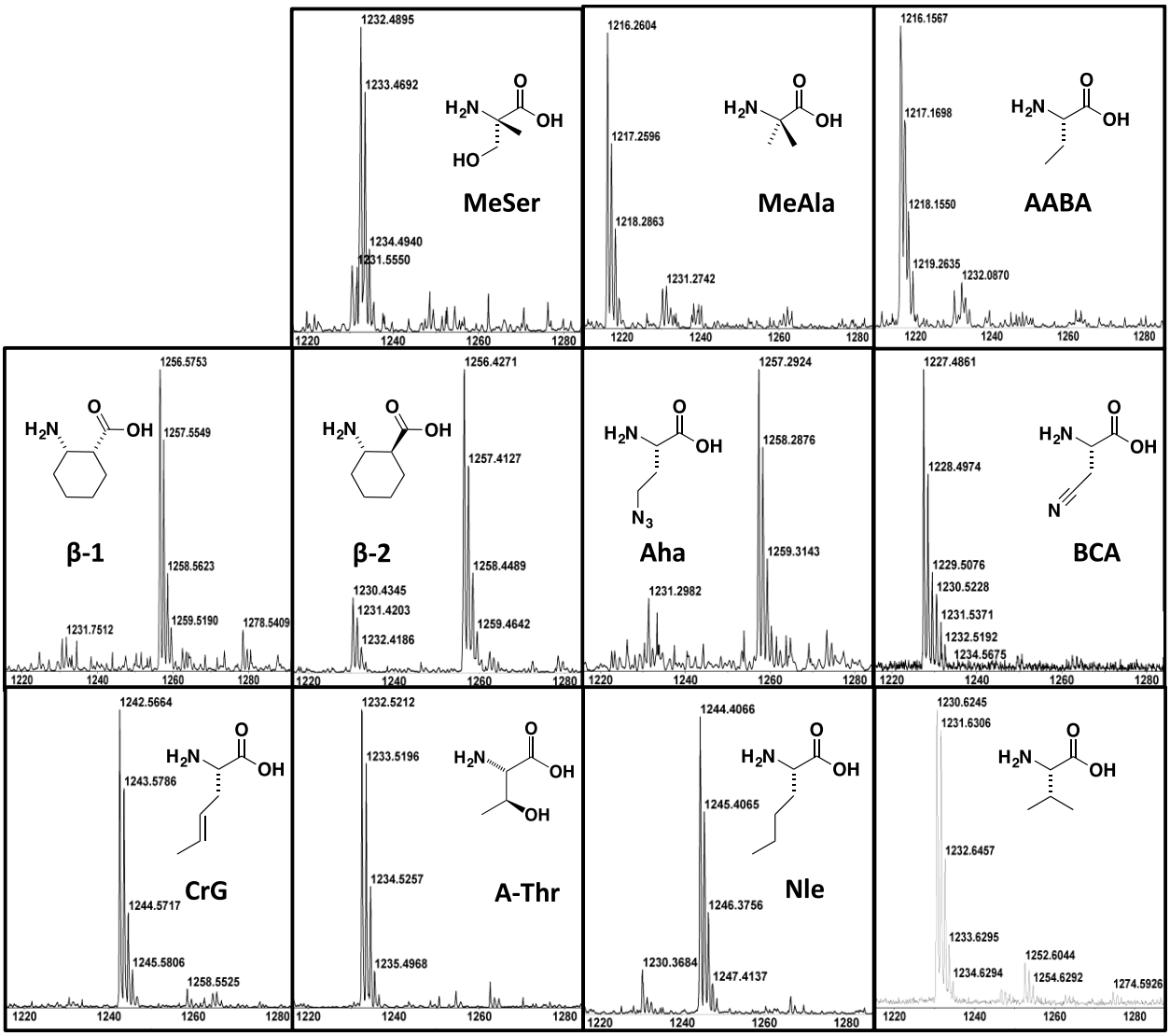

Figure S7. MALDI-TOF MS of in vitro translated peptides showing isotopic resolution.
Translation Assay of each ncAA charged with ValRS T222P. The major peak in each assay corresponds to the derivatized amino acid. In certain cases small peaks from valine contamination are present. See Table S1 for expected masses.

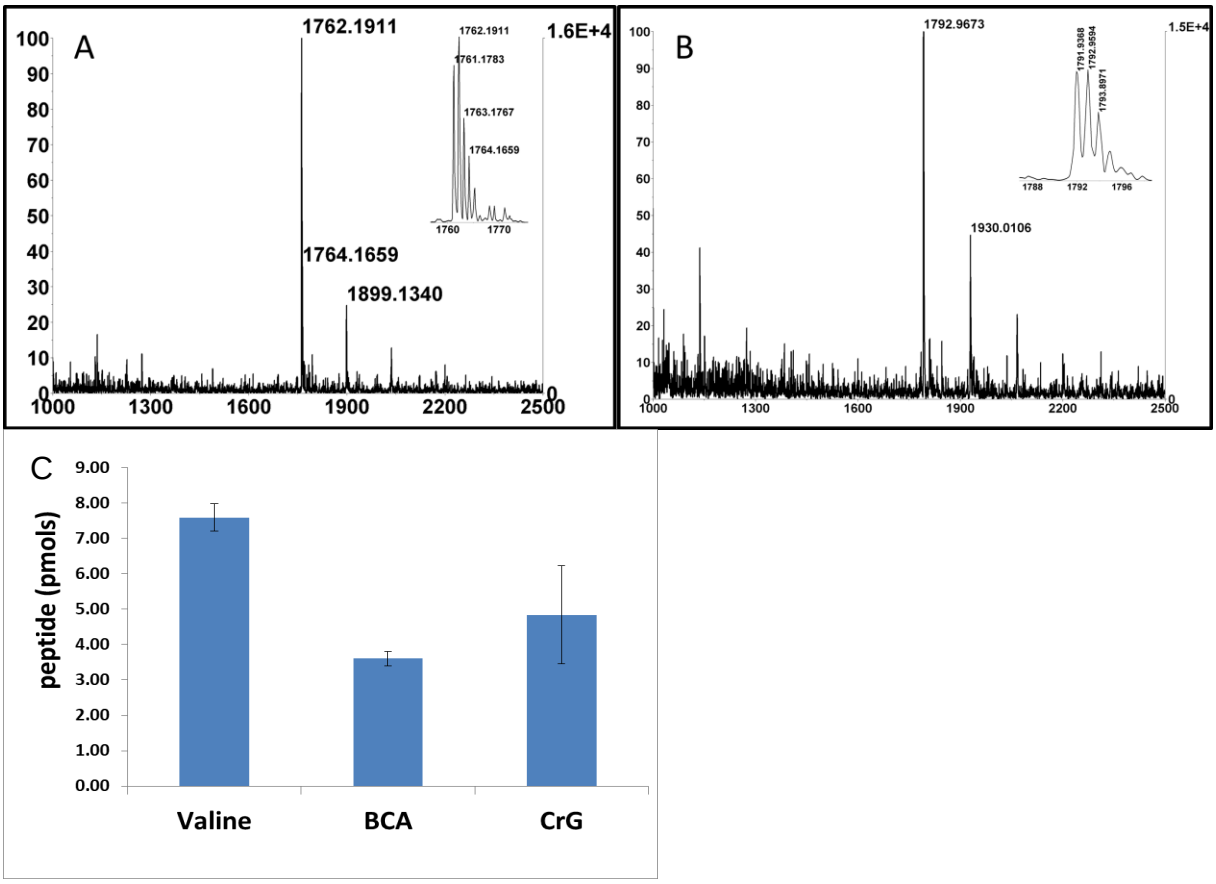

Figure S8. Incorporation of BCA and CrG into template $MH_6MVAAAVEP$. In vitro translations were performed with total tRNA pre-charged with (A) BCA Exp. 1762.7191 Obs 1761.1783 or (B) CrG Exp.1792.7913 Obs 1791.9368. The additional peaks at +137 are occasionally observed in His-rich templates and they are consistent with the sodium trifluoroacetate adduct of the protonated peptide[2]. In (C) the yields of each of the duplicate translation experiments is shown in comparison to a Valine control.

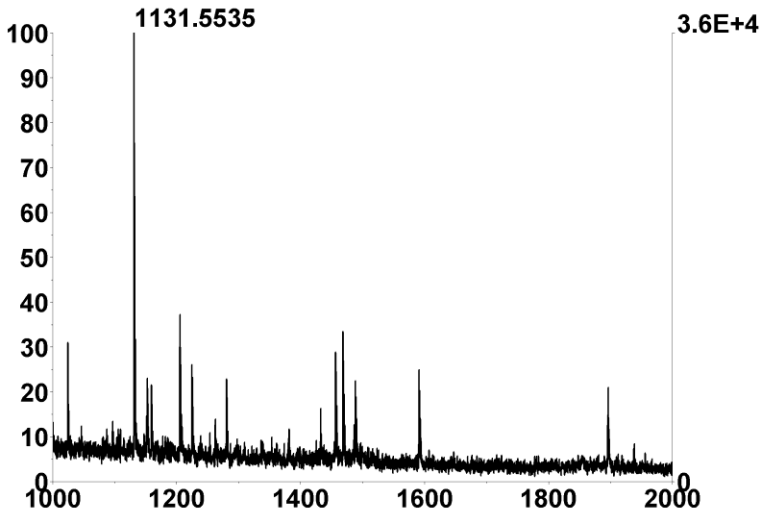

Figure S9. Translations of backbone modified ncAA on N-terminal His-tag templates.
Attempted translation of beta amino acids on longer mRNA templates ($MH_6M\underline{V}EP$) results in truncation. The common product detected was the MH_6M product. Expected *m/z* 1131.447. Observed *m/z* 1131.554.

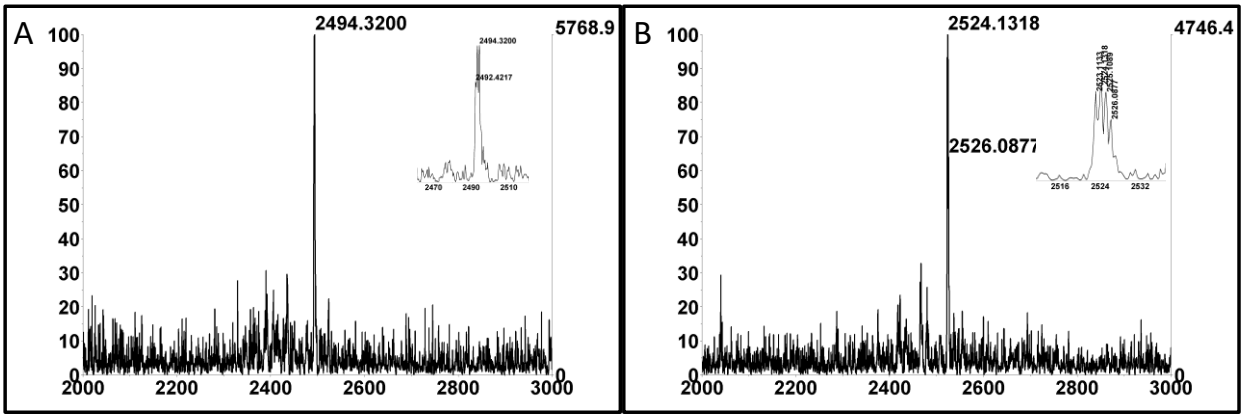

Figure S10. Incorporation of MeAla and MeSer on long template. Incorporation of MeAla (A) and MeSer (B) into an mRNA template coding for peptide sequence MVTNPDCFGNPVCGGGHHHHHH. Peptides were cyclized with dibromoxylene at the two cysteine residues as previously described.[3] (A) Expected *m/z*: 2491.9939, Observed *m/z*: 2492.4217. (B) Expected *m/z*: 2523.9837, observed *m/z*: 2523.1133.

Materials/Methods

General procedures

Matrix-assisted laser desorption/ionization analysis was performed on an Applied Biosystems Voyager by using delayed extraction in reflector positive mode. All spectra were calibrated internally for the AARS screening assay to 756.2352 (the mass of the 4-formylphenoxypropyl triphenylphosphonium AMP derivative). Deacylation of tRNA was performed by dissolving *E. coli* total tRNA (100 mg/mL) (Sigma) in 1M Tris-HCl (pH 9.0), incubation at 37°C for 2 h followed by overnight dialysis against 50 mM Tris-HCl (pH 9.0). The tRNA was recovered by addition of 0.1 volume KOAc (3.0 M pH 5.5) and 4 volumes of cold 100% ethanol. After centrifugation, the tRNA pellet was washed twice with 70% cold ethanol and suspended in dd$H_2$0 at a concentration of 100 mg/mL, aliquoted and stored at -80°C. CCA tRNA nucleotidyltransferase was expressed as described[4].

Sources of Amino Acids

Successful Analogs: α-methyl Serine (MP Biochemicals), α-methyl Alanine (Sigma), aminobutyric acid (Sigma), azidohomoalanine (gift from the Tirrell Lab at Cal Tech), β-cyano Alanine (Bachem), crotylglycine (Chem Service Inc), L-*allo*-Threonine (Sigma), Norleucine (Bachem), L-Valine (Sigma).

Unsuccessful Analogs: α-methyl Glutamic Acid (Chem-Impex), α-methyl Valine (Chem-Impex), L-Homoserine (Chem-Impex), N-methyl L-Serine (Chem-Impex), N-Methyl L-Threonine (Chem-Impex), Hexafluoro-L-Valine (Oakwood Products Inc.), L-Glutamic Acid-γ-hydrazide (Sigma), L-Glutamic Acid-γ-methyl ester (Bachem).

All β amino acids were a gift from Dr. Sam Gellman (University of Wisconsin-Madison).

Synthesis of activated amino acids

N-boc-L-valine (ChemImpex) and N-boc-L-cis-2-aminocyclohexanecarboxylic acid (ChemImpex) were activated with 3,5-dinitrobenzyl chloride (DNB) (Sigma-Aldrich) and deprotected as described [5]. In brief, 0.6 mmol of the amino acid and 0.5 mmol of DNB were added to a dry flask, dried under argon, and dissolved in a solution of dry triethylamine (1 mmol, 139 μL) in DMF (500 μL). The reaction was allowed to proceed under argon for 16 hours at rt. The reaction was then diluted with ether (45 mL) , washed with 0.5 M HCl (3 x 15 mL), saturated $NaHCO_3$ (3 x 15 mL), and saturated NaCl (1 x 15 mL). The organic layer was extracted and dried with $MgSO_4$ before concentration on a rotary evaporator. The resulting oil was incubated for 20 min in 3M HCl/ethyl acetate (3 mL) at rt and subsequently concentrated on a rotary evaporator. The product was washed with ether (3 x5 mL) and

dried on a rotary evaporator. The final product was assessed for purity via ^{1}H NMR and stored as a dry solid at -20°C.

Valine, 3,5-dinitrobenzyl ester. ^{1}H NMR (400 MHz, CD_3OD) δ 9.00 (1H, t, *J* = 2.0 Hz), 8.71 (2H, d, *J* = 2.0 Hz), 5.59-5.51 (2H, m), 4.09 (1H, d, *J* = 4.7 Hz), 2.40-2.32 (1H, m), 1.10-1.07 (6H, m).
(1R,2S)-2-aminocyclohexane-1-carboxylate, 3,5-dinitrobenzyl ester. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 8.81 (1H, t, *J* = 2.1 Hz), 8.72 (2H, d, *J* = 2.1 Hz), 5.46-5.37 (2H, m), 3.08 (1H, ddd, *J* = 4.0 Hz , *J* = 4.2 Hz, *J* = 7.7 Hz), 2.03-1.92 (1H, m), 1.87-1.57 (5H, m), 1.48-1.34 (3H, m).

ValRS T222P Expression and Purification

The ValRS T222P construct was made by digesting a pET24a vector containing the wild type ValRS with StuI and Eam1105I. The resulting linear plasmid was purified via 1% agarose gel electrophoresis and a commercially ordered gene fragment (see below) containing the T222P mutation was ligated into the linear plasmid using T4 DNA ligase. The resulting plasmid was verified using DNA sequencing (Eurofins), transformed into chemically competent BL21 *E. coli* cells, and grown overnight on ampicillin LB plates. Single colonies were picked and grown in an overnight 5 mL culture with ampicillin at 37°C. This starter culture was diluted 1:100 into fresh 200 mL LB media with ampicillin and grown to mid-log phase. Protein production was induced upon addition of 0.10 mM final concentration of IPTG and grown for 3 h at 37°C. Cells were pelleted and lysed with 10 mL of B-PER and 50 µL histidine-tagged compatible protease inhibitor cocktail (Sigma P8849). The lysate was centrifuged at 15,000 rpm and the soluble fraction was applied to a pre-equilibrated Ni-NTA Agarose (McLab) column and allowed to bind for 1 h at 4°C. The column was washed with two 10 mL portions of wash buffer (20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 10 mM $Mg(OAc)_2$, 5 mM BME), and eluted into 5 x 1 mL fractions of elution buffer (20 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, 10 mM $Mg(OAc)_2$, 5 mM BME). Fractions containing protein were pooled and dialyzed overnight into enzyme storage buffer (50 mM HEPES-KOH, 100 mM KCl, 10 mM $MgCl_2$, 7 mM BME, 30% Glycerol). 2.5 µL of post dialysis protein was mixed with 2.5 µL of 2x SDS Lamelli Sample Buffer and was run on a 10% SDS-Page gel to verify molecular weight.

Gene Fragment T222P Mutation

5'-CGA TGG ACG A<u>AG GCC T</u>GT CCA ATG CGG TGA AAG AAG TTT TCG TTC GTC TAT ATA AAG AAG ACC TGA TTT ACC GTG GCA AAC GCC TGG TAA ACT GGG ACC CGA AAC TGC GCA CCG CTA TCT CTG ACC TGG AAG TGG AAA ACC GCG AAT CGA AAG GTT CGA TGT GGC ACA TCC GCT ATC CGC TGG CTG ACG GTG CGA AAA CCG CAG ACG GTA AAG ATT ATC TGG TGG TCG C<u>GA CTC CCC GTC</u> CGG AAA CCC G-3' (Cloning restriction sites are underlined).

Valine depletion in ValRS T222P

To limit the contamination of valine purified with the ValRS T222P enzyme we first performed a mock tRNA pre-charging performed exactly as above but lacking any amino acid. The synthetase was then re captured on Ni-NTA resin and dialyzed into enzyme storage buffer.

MALDI-AARS Screening Assay Procedure

Each charging reaction contained 30 mM HEPES-KOH (pH 7.4), 15 mM $MgCl_2$, 25 mM KCl, 2 mM 2-mercaptoethanol, 6 mM ATP, 0.09 mg/mL BSA (previously dialyzed into ddH_2O), 0.02 units/μL PP_iase, 350 μM deacylated tRNA, 5 μM ValRS T222P, 10 mM non-canonical amino acid, and ddH_2O to a final volume of 50 μL. Each reaction was initiated by addition of ValRS T222P to the mixture and incubated for 30 minutes at 37°C. Each reaction was quenched by addition of 0.1 volume of NaOAc (3.0 M, pH 5.2) and tRNA was extracted with unbuffered phenol:chloroform:isoamyl alcohol solution (25:24:1). The aqueous layer was subjected to a second extraction with chloroform, followed by ethanol precipitation (3 vol.) with 0.1 volume NaOAc (3.0 M, pH 5.2). After centrifugation, the pellet was washed twice with 70% cold ethanol and resuspended in 200 mM NaOAc (pH 5.0) for use in AARS screening assay. tRNA for use in screening assay (6.75 μL) was added to water (3.75 μL), 30 mM (4-formylphenoxyproyl)triphenylphosphonium bromide in MeOH, 20 mM $NaCNBH_3$ in 50 mM NaOAc (pH 5.0) and tumbled at 37°C for 2 h. The reaction was quenched with 0.1 volume 4.4 M NH_4OAc pH 5.0 and 3 volumes of ice cold 100% EtOH, pelleted and washed twice with 70% EtOH and twice with 100% EtOH. The resulting pellet was resuspended in 2.25 μL 200 mM NH_4OAc and digested with 0.25 μL of 1 U/μL of Nuclease P1 (in 200 mM NH_4OAc, pH 5.0). After 20 minutes at room temperature, the reaction was quenched by placing on ice. One microliter was mixed with saturated CHCA (α-cyano-4-hydroxycinnamic acid) matrix in 1:1 MeCN:1% TFA (9 μL). One microliter of the resulting suspension was spotted on a MALDI plate and analyzed.

tRNA Pre-charging

The MALDI-AARS screening assay procedure was followed up until the first ethanol precipitation step. The precipitated tRNA was then dissolved in 20 μL 5 mM KOAc (pH 5.5) and stored at -80 °C until use.

$tRNA^{Val}$ PCR and Purification

DNA encoding for tRNA transcripts were prepared using PCR primers (Integrated DNA Technologies) from table S2. Primer extension was performed by mixing V-1 and V-2 (1 μM each) in Q5 polymerase buffer as per manufacture protocol (NEB M0491S) on a 10 μL scale. PCR was performed by denaturing (95°C for 1 min) following by annealing (50°C for 1 min) and extension (72°C for 1 min) for 5 cycles. The resulting PCR was diluted into 200 μL PCR buffer and amplified using V-3 and V-4 primers (95°C for 40 sec, 62°C for 40 sec, 72°C for 40 sec, 5 cycles). The final PCR was performed on a 1 mL scale (10 x 100 μL) using 5 μL of the previous PCR (95°C for 40 sec, 63°C for 40 sec, 72°C for 40 sec, 15 cycles). The final product

was verified by 3% agarose gel analysis with ethidium bromide staining. The resulting DNA was purified by phenol/chloroform extraction followed by ethanol precipitation and dissolved in 50 μL water.

T7 Transcription

Transcription of tRNA was done by incubating the entirety of the PCR amplified DNA or synthetic template primers (Table S4, 1 μM each) in transcription buffer (40 mM Tris-HCl pH 7.8, 0.01% v/v Triton X-100, 2.5 mM spermidine, 25 mM MgCl2, 10 mM DDT, 5 mM each NTP, 5 mM GMP, 0.2 units/mL RNAsin, 1 U/mL PPi, 0.1 mg/mL T7 polymerase) on a 1 mL scale and incubated overnight at 37°C. The reaction was quenched by addition of 100 μL 0.5 M EDTA (pH 8.0) and 435 mg urea (8 M final concentration), heated to 90 °C for 5 minutes, and loaded onto and purified by a 10% denaturing urea page gel. The desired band was visualized by UV shadowing, extracted by crushing and soaking in 0.3 M NaCl or electroelution followed by ethanol precipitation. Activity of tRNA was verified by performing a charging assay with transcribed tRNA, wild type ValRS, and valine. A slightly modified protocol was used for T7 transcription of dFx as described[6].

^{32}P-labeling of tRNA using CCA tRNA nucleotidyl transferase

The 3' end of tRNA was labeled with ^{32}P (α^{32}P-ATP, Perkin Elmer) in 100 μL reactions as described[7]. The tRNA was purified by extraction in 1 volume of 25:24:1 buffered phenol:chloroform:isoamyl alcohol. The mixture was vortexed and centrifuged for 1 min at 13000 rpm. The aqueous layer was then added to 1.1 volumes of chloroform, vortexed, and centrifuged. The aqueous layer was loaded onto a NAP-5 column (GE Healthcare) pre-equilibrated with water, followed by addition of 400 μL of water. Labeled tRNA was eluted with 500 μL of water into a clean microcentrifuge tube. The eluent was added to a PL-10 centricon (Millipore) and centrifuged at 14000 rpm for 30min, pausing at 15 min to ensure the membrane had not dried out. The membrane was then washed with 250 μL of water and centrifuged at 14000 rpm for 30 min. The filter was then removed, inverted on a clean microcentrifuge tube, and eluted by centrifugation at max speed for 1 min. 1 μL of the product was removed for analysis of radioactivity via scintillation counting. The remainder was stored in an acrylic shielded box at -20°C.

^{32}P Charging Assay using ValRS T222P

Aminoacylation of $tRNA^{Val}$ with ValRS T222P was adapted from Wolfson and Uhlenbeck[7]. 20 μL reactions containing 30 mM HEPES-KOH (pH 7.4), 20 μM unlabeled $tRNA^{Val}_{GUA}$, 25000 cpm ^{32}P-labeled $tRNA^{Val}_{GUA}$, 15 mM $MgCl_2$, 25 mM KCl, 2 mM ATP, 0.09mg/mL dialyzed BSA, 100 μM L-valine or 10 mM L-cis-2-aminocyclohexanecarboxylic acid, and 156 nM ValRS T222P were prepared. Water, HEPES buffer, and the tRNA species were added to a 96-well plate, heated to 95°C for 3 min, and cooled to 25°C at a rate of 0.2°C/s. $MgCl_2$, KCl, ATP, and BSA were added and the reaction was heated for 5 minutes at 25°C and then placed on ice. The amino acids and synthetase were added to the ValRS reaction and

incubated at 37°C. At 0, 2, 5, 20, 60, and 120 minutes, 1 μL of each reaction was removed and added to 4 μL of 0.1U/μL Nuclease P1 (Wako) in 200mM NaOAc (pH 5) and kept on ice. Once the final timepoint was collected, the nuclease reactions were incubated for 10 minutes at 25°C. 1 μL of each well was then spotted on 10 cm tall PEI cellulose plates (Sigma) pre-run in water and allowed to dry. The plates were run in 5:10:85 acetic acid:saturated boric acid:water, allowed to dry, and wrapped in saran wrap before exposure to a phosphorimaging screen (GE Healthcare and Life Sciences) overnight. The screen was imaged on a GE Typhoon 9410 scanner and the ratio of aminoacylated tRNA to nonacylated tRNA was quantified using density analysis on ImageQuant5. Reactions were run in duplicate.

^{32}P Charging Assay using dFx

20 μL reactions containing a final concentrations of 200 mM HEPES-KOH (pH 7.5), 20 μM $tRNA^{Val}_{GUA}$, 25000 cpm labeled $tRNA^{Val}_{GUA}$, 0.6 M $MgCl_2$, 20 μM dFx, and 5 mM DNB-valine or 10 mM DNB-cis-2-aminocyclohexanecarboxylic acid were prepared. Water, HEPES buffer, and the tRNA species were added to a 96-well plate, heated to 95°C for 3 min, and cooled to 25°C at a rate of 0.2°C/s. $MgCl_2$ and dFx were added to the flexizyme reactions, and the reactions were incubated for 5 minutes at 25°C and then placed on ice. Activated amino acids were added to the flexizyme reaction and allowed to proceed on ice. At 0, 2, 5, 20, 60, and 120 minutes, 1 μL of each reaction was removed and added to 4 μL of 0.1U/μL Nuclease P1 (Wako) in 20 0mM NaOAc (pH 5) and kept on ice. The nuclease reaction and subsequent analysis are as above.

Mutant Ribosome Isolation

A plasmid containing a mutant 23S ribosome subunit in a pLK35 vector (gifted by Shepeartz Lab, Yale University) was transformed into RNAse deficient A19 strain of *E. coli*. A 2 mL starter culture was diluted 1:5000 into 1 L of LB and grown to OD 0.60 at 37 °C followed by 2 hours at 42 °C for induction of the λ P_L promoter in the pLK35 plasmid. From here, ribosome isolation was performed as previously reported.[8]

References:

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Compound NMRs