

Electronic Supplementary Information

for

Enzymatic *N*-methylaminoacylation of tRNA using chemically misacylated AMP as a substrate

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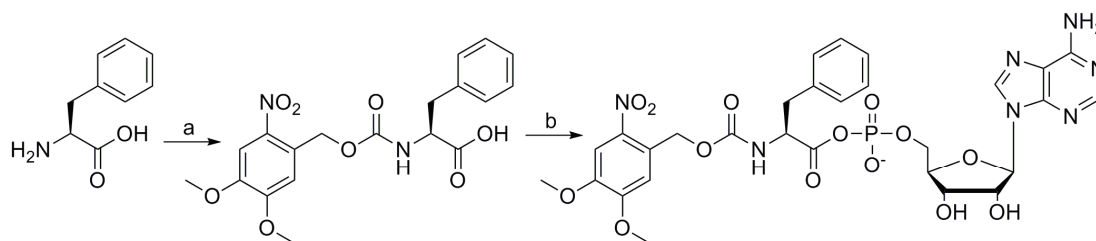
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1. General. Reagents and solvents were purchased from standard suppliers and used without further purification. ^1H NMR and ^{13}C NMR spectra were taken with a JEOL JNM-A500 (500 MHz) and a JEOL JNM-A400 (400 MHz), respectively. FAB mass spectra were recorded on a JEOL JMS HX-110A spectrometer. DNA oligomers were purchased from Gene Design Inc. (Japan).

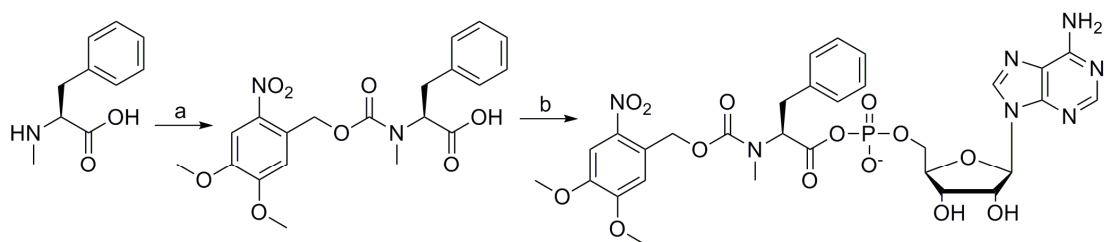
2. Synthesis of Chemically Misacylated AMP.



Scheme 1 (a) 6-nitroveratryloxycarbonyl chloride, Na_2CO_3 , dioxane, H_2O , 39%; (b) AMP, DCC, pyridine, HCl, H_2O .

Preparation of *N*-NVOC-phenylalanine (Scheme 1-a). L-Phenylalanine (500 mg, 3.03 mmol) and sodium bicarbonate (321 mg, 3.03 mmol) were dissolved in 10 mL of H_2O . To the solution was added 6-nitroveratryloxycarbonyl chloride (NVOC-Cl, 838 mg, 3.04 mmol) in 10 mL of dioxane. The resulting mixture was stirred overnight at room temperature and the solvent was evaporated. The resulting products were re-suspended in CH_2Cl_2 and washed with 1 M aqueous sodium bisulfate and then with brine, dried over magnesium sulfate, and concentrated in vacuo. The crude product was recrystallized from hexane/ethyl acetate to give *N*-NVOC-phenylalanine (39%): $\nu_{\text{max}}/\text{cm}^{-1}$ 3338, 1760, 1700, 1515, 1065, 874; ^1H NMR (CDCl_3 , 500 MHz) δ 7.69 (1H), 7.16–7.31 (5H), 6.90 (1H), 5.51 (2H), 5.23 (1H), 4.70 (1H), 3.93 (3H), 3.88 (3H), 3.12 (2H); ^{13}C NMR (CDCl_3 , 400 MHz) δ 175.3, 155.4, 153.6, 148.1, 139.7, 135.3, 129.2, 128.8, 127.8, 127.4, 109.9, 108.2, 64.0, 56.4, 54.5, 37.6; HRMS (FAB) m/z calcd. for $\text{C}_{19}\text{H}_{20}\text{N}_2\text{O}_8$ $[(\text{M})^+]$ 404.1220, found 404.1227.

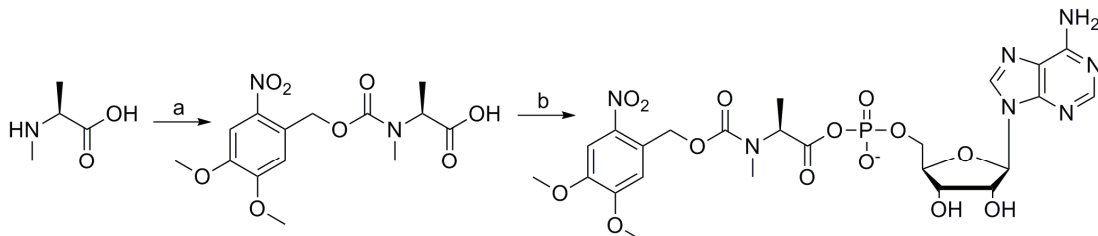
Preparation of *N*-NVOC-phenylalanyl-AMP (Scheme 1-b). To the solution of *N*-NVOC-phenylalanine (15 mg, 0.037 mmol) in pyridine (500 μL) were added AMP (15 mg, 0.043 mmol), H_2O (32 μL), 1N HCl (45 μL), and DCC (210 mg, 1.02 mmol) and the resulting mixture was stirred for 3 h at 4 $^\circ\text{C}$. Crude products were precipitated upon addition of cold acetone (14 mL) and collected by centrifugation, washed with cold acetone/EtOH (1/1, 14 mL), and then purified by reverse-phase HPLC on a Wakosil 5C18 column using linear gradient of acetonitrile in 0.1 M NH_4OAc buffer, pH 4.5. *N*-NVOC-phenylalanyl-AMP was redissolved in DMSO to give a 1 mM solution. MALDI-TOF m/z calcd. for $[(\text{M} - \text{H})^-]$ 732.17, found 732.14.



Scheme 2 (a) 6-nitroveratryloxycarbonyl chloride, Na₂CO₃, dioxane, H₂O, 32%; (b) AMP, DCC, pyridine, HCl, H₂O.

Preparation of *N*-NVOC-*N*-methylphenylalanine (Scheme 2-a). *N*-NVOC-*N*-methylphenylalanine was obtained in a similar manner as *N*-NVOC-phenylalanine with slight modifications as follows. The crude product was purified by PLC and GPC instead of recrystallization to give *N*-NVOC-*N*-methylphenylalanine (32%): $\nu_{\max}/\text{cm}^{-1}$ 2939, 1684, 1582, 1522, 1275, 1065, 868; ¹H NMR (CDCl₃, 500 MHz at 60 °C) δ 7.66 (1H), 6.98–7.06 (5H), 6.78 (1H), 5.37 (2H), 4.50 (1H), 3.84 (3H), 3.70 (3H), 3.07–3.38 (2H), 2.71 (3H); ¹³C NMR (CDCl₃, 400 MHz) δ 177.4, 156.8, 153.4, 149.5, 147.6, 139.65, 128.7, 128.3, 128.2, 126.0, 109.5, 107.8, 64.1, 58.4, 56.2, 35.1, 18.4; HRMS (FAB) m/z calcd. for C₂₀H₂₃N₂O₈ [(M + H)⁺] 419.1454, found 419.1446.

Preparation of *N*-NVOC-*N*-methylphenylalanyl-AMP (Scheme 2-b). *N*-NVOC-*N*-methylphenylalanyl-AMP was obtained in a similar manner as *N*-NVOC-phenylalanyl-AMP: MALDI-TOF m/z calcd. for [(M – H)[–]] 746.18, found 746.16.



Scheme 3 (a) 6-nitroveratryloxycarbonyl chloride, Na₂CO₃, dioxane, H₂O, 46%; (b) AMP, DCC, pyridine, HCl, H₂O.

Preparation of *N*-NVOC-*N*-methylalanine (Scheme 3-a). *N*-NVOC-*N*-methylalanine was obtained in a similar manner as *N*-NVOC-phenylalanine with slight modifications as follows. The crude product was purified by PLC instead of recrystallization to give *N*-NVOC-*N*-methylalanine (46%): $\nu_{\max}/\text{cm}^{-1}$ 2941, 1684, 1580, 1522, 1275, 1065, 868; ¹H NMR (CDCl₃, 500 MHz) δ 7.61 (1H), 6.90 (1H), 5.51 (1H), 5.48 (1H), 4.74 (1H), 3.87 (6H), 2.90 (3H), 1.41 (3H); ¹³C NMR (CDCl₃, 400 MHz) δ 176.2, 156.2, 153.7, 148.2, 139.8, 127.9, 110.2, 108.4, 64.5, 56.4, 54.5, 30.8, 14.8; HRMS (FAB) m/z calcd. for C₁₄H₁₈N₂O₈ [(M)⁺] 342.1063, found 342.1054.

Preparation of *N*-NVOC-*N*-methylalanyl-AMP (Scheme 3-b). *N*-NVOC-*N*-methylalanyl-AMP was obtained in a similar manner as *N*-NVOC-phenylalanyl-AMP: MALDI-TOF m/z calcd. for [(M – H)[–]] 670.15, found 670.29.

3. Enzymatic Aminoacylation of tRNA.

Run-off transcription of *E. coli* tRNA^{Phe}. DNA templates for transcription were obtained by PCR reaction using *Pfu Ultra* HF DNA polymerase (Stratagene) from template ODN 5'-(GCC GGG GAT CCT AAT ACG ACT CAC TAT AGC GCG GAT AGC TCA GTC GGT AGA GCA GGG GAT TGA AAA TCC CCG TGT CCT TGG TTC GAT TCC GAG TCC GCG CAC CA)-3' using 1.0 μ M of forward primer 5'-(GCC GGG GAT CCT AAT ACG ACT CA)-3' and 1.0 μ M of reverse primer 5'-(**UGG** TGC GCG GAC TCG GAA TCG)-3', in which the two 5'-nucleotides (in bold) were modified with C2'-methoxy (-OCH₃) to inhibit non-templated nucleotide addition at the 3'-end of the RNA by T7 RNA polymerase.¹ Transcription was carried out in T7 MEGashortscript kit (Ambion) directly using the PCR solution (typically 6 μ L PCR solution in 40 μ L transcription reaction). The transcribed tRNA was purified by denaturing PAGE (8%). The tRNA obtained was further purified using Microcon YM-30 (Millipore) and G-25 Microspin Columns (GE Healthcare).

Expression of *E. coli* PheRS. His₆-tagged *E. coli* PheRS was expressed using M15[pREP4] strain harboring the plasmid pQE30, encoding the *E. coli* PheRS under the control of IPTG promoter (gift from Prof. T. Ueda of the University of Tokyo). The expressed *E. coli* PheRS was purified using Ni-NTA column (Qiagen) under native conditions following the supplier's manual and dialyzed. The concentration of purified *E. coli* PheRS was determined using the typical Bradford assay and concentration of PheRS was determined as $\alpha_2\beta_2$ unit.

Photodeprotection of NVOC-protective group. NVOC-protected aa-AMP (1 mM in DMSO) was mixed with the same volume of KOAc (1 mM, pH 5.0) and H₂O. The mixture was photo-irradiated for 5 min on ice using a Xenon Light Source MAX-301 with LX filter (Asahi Spectra, 365 nm, 100% intensity). The resulting mixture was directly used without further purification.

Enzymatic Aminoacylation of tRNA^{Phe}. Typical aminoacylation reaction was carried out at 37 °C in 10 μ L of 100 mM Tris-HCl buffer (pH 7.5) containing 15 mM MgCl₂, 40 mM KCl, 1 mM DTT, 0.04 unit/ μ L PPIase, 4 μ M tRNA^{Phe}, and 0, 50, or 500 nM *E. coli* PheRS in the absence or presence of amino acid (100 or 1000 μ M) with ATP (4 mM) or photo-deprotected NVOC-aa-AMP (~100 μ M). After incubation, the mixture was diluted with 90 μ L of 0.3 M aqueous sodium acetate (pH 5.2). The solution was extracted with 100 μ L of phenol/chloroform/isoamyl alcohol (25/24/1), followed by ethanol precipitation and rinse with cold 75% (v/v) aqueous ethanol, dried in vacuo, and resuspended in a mixture of 2.5 μ L of 0.3 M aqueous sodium acetate (pH 5.2) and 2.5 μ L of loading buffer (50% glycerol in 0.2 M sodium acetate pH 4.5). The samples were applied on 9% acid PAGE (running buffer: 0.1 M sodium acetate buffer pH 5.2) at 4 °C and aminoacylation efficiencies were checked by staining with SYBR Green II.

4. Translation and Mass Analysis.

mRNA Template. DNA templates for transcription were obtained by PCR reaction using *Pfu Ultra* HF DNA polymerase (Stratagene) from template ODN 5'-(AAG GAG ATA TAC CAA TGG ACT ACA AGG ATG ACG ATG ACA AGC AAA AAC TGT TCC TGA CGC ATT AG)-3' using 0.2 μ M of forward primer 5'-(GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC ACA ACG GTT TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACC AAT GGA C)-3' and 0.2 μ M of reverse primer 5'-(TAT TCA CTA ATG CGT CAG GAA CAG)-3'. The resulting double-stranded template DNA contains a T7 promoter, an SD sequence, an initiation codon (ATG), and FLAG-tag sequence. The mRNA was obtained by T7 run-off transcription of this template using T7 MEGAshortscript kit (Ambion).

Translation, Purification, and Mass Analysis of Oligopeptide. Translation (PureSystem-classic II custom PheRS and Phe minus, Post Genome Institute Co., Ltd.) was initiated by the addition of 10 μ g of the mRNA template with misacylated tRNA^{Phe} (10 μ g, purified after enzymatic aminoacylation) (total reaction volume, 50 μ L). The translation mixture was incubated at 37 °C for 60 min. To this mixture was added 45 μ L of anti-FLAG M2 affinity gel (Sigma, prewashed twice with 1.3 mL of TBS buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) and resuspended in 350 μ L of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20)). The mixture was gently inverted for 4 h at 4 °C, applied to a MicroSpin column (GE Healthcare), and filtered by centrifugation. The agarose beads were washed five times with 300 μ L of prechilled TBS buffer and resuspended in 170 (100 + 70) μ L of elution buffer (0.1 M glycine HCl, pH 3.5). The suspension was incubated with gentle shaking for 5 min at room temperature and filtered by centrifugation. To the filtrate was added 17 μ L of 0.5 M Tris-HCl (pH 7.4) containing 1.5 M NaCl, and the resulting solution was dialyzed (Mini Dialysis Kit, 1 kDa cut off; GE Healthcare) against a large volume of distilled water for 24 h to purify the oligopeptide. The resulting solution was lyophilized and dissolved in 10 mg/mL solution of α -cyano-4-hydroxycinnamic acid dissolved in a 1:1 mixture of water and acetonitrile containing 0.3% trifluoroacetic acid. The resulting mixture was spotted onto a MALDI plate and air-dried. MALDI-TOF mass spectra were measured on a Voyager Elite instrument (Applied Biosystems).

5. Fig. S1

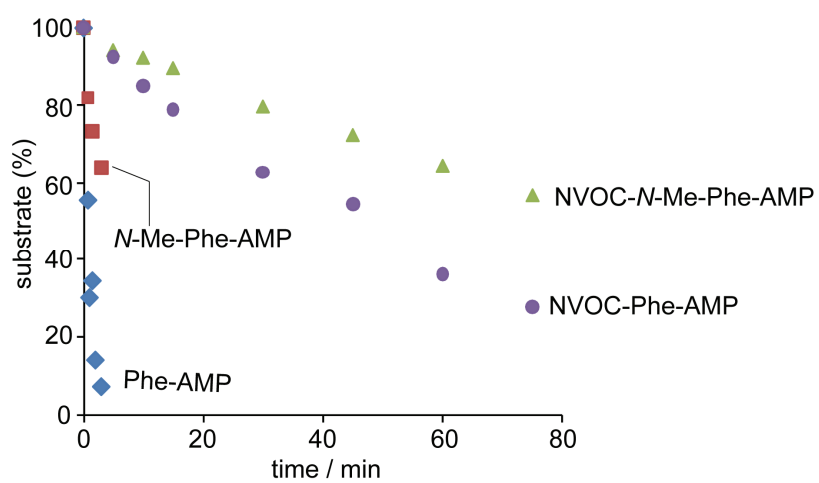


Fig. S1 Hydrolysis of aa-AMP substrates in an acylation buffer (100 mM Tris-HCl, pH 7.5 containing 15 mM MgCl₂, 40 mM KCl, and 1 mM DTT) at 37 °C.

6. Fig. S2

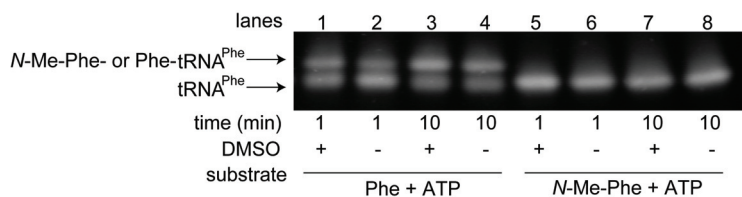


Fig. S2 Effect of DMSO for enzymatic aminoacylation. Aminoacylation reactions of tRNA^{Phe} (4 μM) were carried out with PheRS (50 nM) in 100 mM Tris-HCl buffer (pH 7.5) containing 15 mM MgCl₂, 40 mM KCl, and 0.04 units/μL inorganic pyrophosphatase, 1 mM dithiothreitol, 4 mM ATP with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) DMSO (10% v/v) using 100 μM Phe (lanes 1–4) or 100 μM *N*-Me-Phe (lanes 5–8) as a substrate. Reaction solutions were incubated for 1 (lanes 1, 2, 5, and 6) and 10 (lanes 3, 4, 7, and 8) min, respectively, and acylation yields were evaluated by acid PAGE analysis.

7. Fig. S3

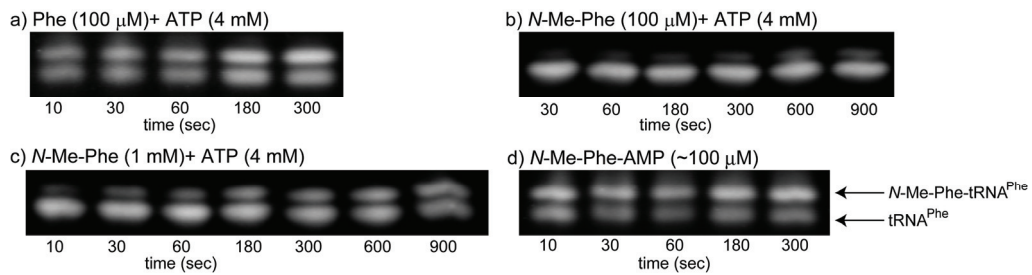


Fig. S3 Aminoacylation with a higher concentration of PheRS. Time-course of aminoacylation reaction of tRNA^{Phe} (4 μ M) with PheRS (500 nM) using a) 100 μ M Phe + ATP, b) 100 μ M *N*-Me-Phe + ATP, c) 1 mM *N*-Me-Phe + ATP, and d) ~100 μ M *N*-Me-Phe-AMP. The reactions were carried out in 100 mM Tris-HCl buffer (pH 7.5) containing 15 mM MgCl₂, 40 mM KCl, 0.04 units/ μ L inorganic pyrophosphatase, 1 mM dithiothreitol, and 10% DMSO at 37 °C.

8. Fig. S4

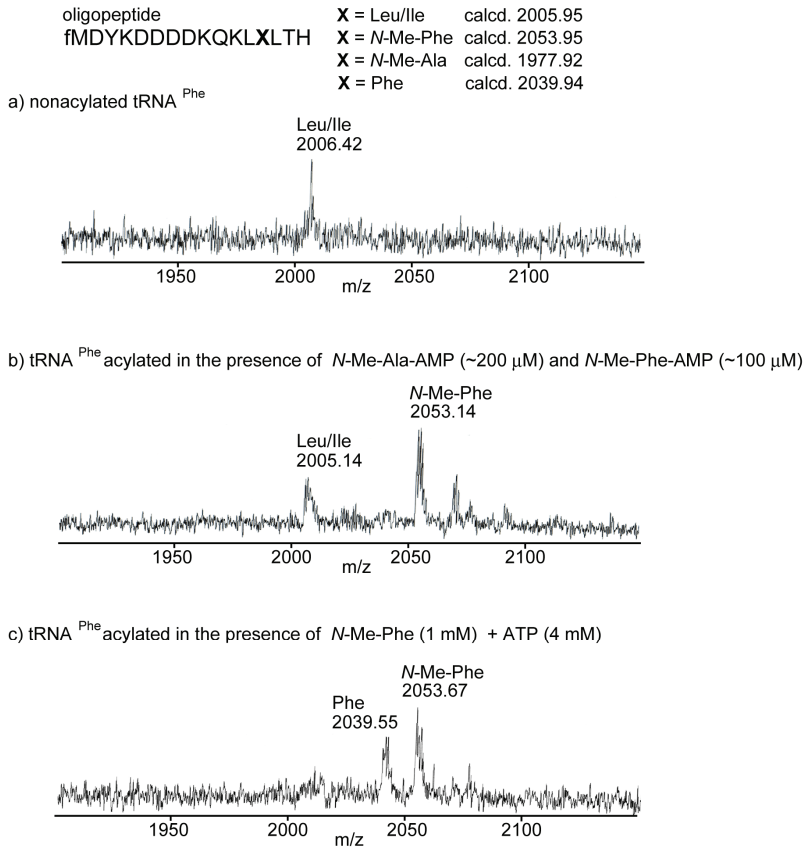


Fig. S4 MALDI TOF mass spectra of the translated 16-mer oligopeptide products using the mRNA template shown in the text in the cell-free translation system (free from PheRS and Phe) in the presence of various forms of tRNA^{Phe}: a) nonacylated tRNA^{Phe} simply treated in the acylation buffer, b) preacylated tRNA^{Phe} using a mixture of *N*-Me-Ala-AMP (200 μ M) and *N*-Me-Phe-AMP (100 μ M) with PheRS (500 nM) for 30 sec at 37 °C, and c) preacylated tRNA^{Phe} using *N*-Me-Phe (1 mM) and ATP (4 mM) with PheRS (500 nM) for 15 min at 37 °C (acylation buffer; 100 mM Tris-HCl (pH 7.5) containing MgCl₂ (15 mM), KCl (40 mM), inorganic pyrophosphatase (0.04 units/mL), DTT (1 mM), and 10% DMSO). Figures S4a, S4b, and S4c respectively show that Leu/Ile can be incorporated at the Phe codon in the absence of Phe and PheRS, that competition is highly in favor of *N*-Me-Phe-AMP over *N*-Me-Ala-AMP, and that a substantial amount of Phe is incorporated probably as a result of Phe contamination in *N*-Me-Phe.

9. Reference

1. C. Cao, M. Zheng and S. Rudisser, *RNA*, 1999, **5**, 1268.