Electronic Supplementary Material (ESI) for Chemical Science

Supporting information for:

Incorporation of electrically charged N-alkyl amino acids into ribosomally synthesized peptides via post-translational conversion

Takashi Kawakami,*[a] Toru Sasaki,[b] Patrick C. Reid,[b] and Hiroshi Murakami*[a]

[a] Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo, 153-8902, Japan, Tel/Fax: +81-3-5465-8850

[b] PeptiDream Inc. 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan, Tel/Fax: +81-3468-9022

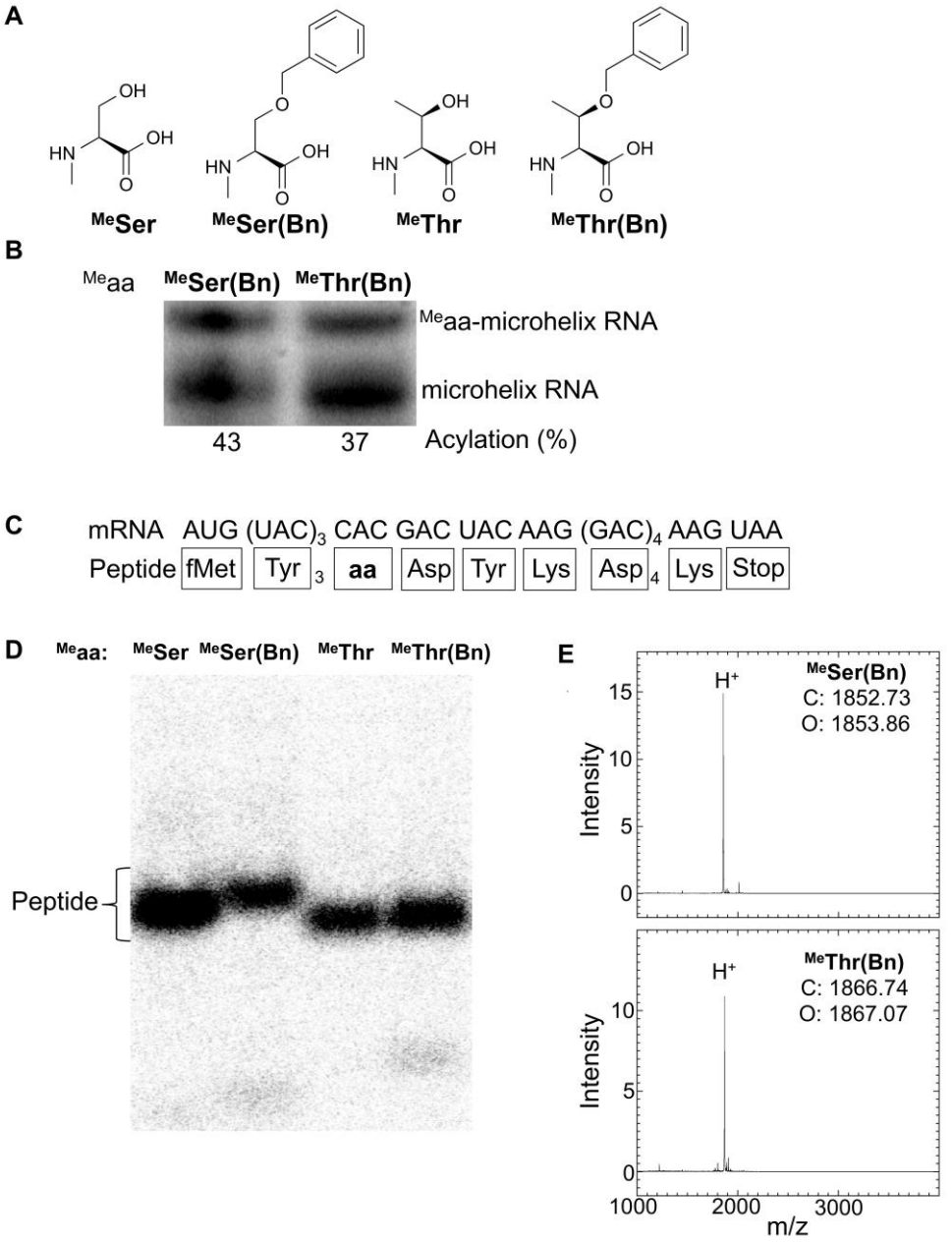


Fig. S1 Ribosomal incorporation of *O*-benzyl-*N*-methyl-serine and *O*-benzyl-*N*-methyl-threonine into a peptide using flexizyme-catalyzed tRNA aminoacylation. (A) Chemical structures of *N*-methyl-serine (^{Me}Ser), *O*-benzyl-*N*-methyl-serine [^{Me}Ser(Bn)], *N*-methyl-threonine (^{Me}Thr), and *O*-benzyl-*N*-methyl-threonine [^{Me}Thr(Bn)]. (B) Acid urea-PAGE of microhelix RNAs aminoacylated with the *O*-benzyl-*N*-methyl amino acid by flexizyme. The *O*-benzyl-*N*-methyl amino acids were charged onto a microhelix RNA using the dinitro-flexizyme (dFx) and the corresponding *O*-benzyl-*N*-methyl amino acid 3,5-dinitrobenzyl esters. The yields of the *N*-methyl aminoacyl-(^{Me}aa-)microhelix RNAs were calculated based on the fluorescence intensity of the ^{Me}aa-microhelix RNAs (I) and the microhelix RNAs (II) using the expression (I) / [(I) + (II)] (C) Sequences of mRNA and peptides encoded in the mRNA used for *N*-methyl amino acid incorporation. (D) Tricine-SDS-PAGE of the expressed peptides labeled with [^{14}C]-Asp, which were detected by autoradiography. Peptides were expressed in the presence of the designated *N*-methyl aminoacyl-tRNA$^{Asn\text{-}E2}_{GUG}$ prepared using flexizyme. (E) MALDI-TOF mass spectra of the *O*-benzyl-*N*-methyl amino acid-containing peptides. Calculated molecular mass (C:) and observed molecular mass of the major peak (O:) for singly charged species $[M + H]^{+1}$ are shown in each spectrum.

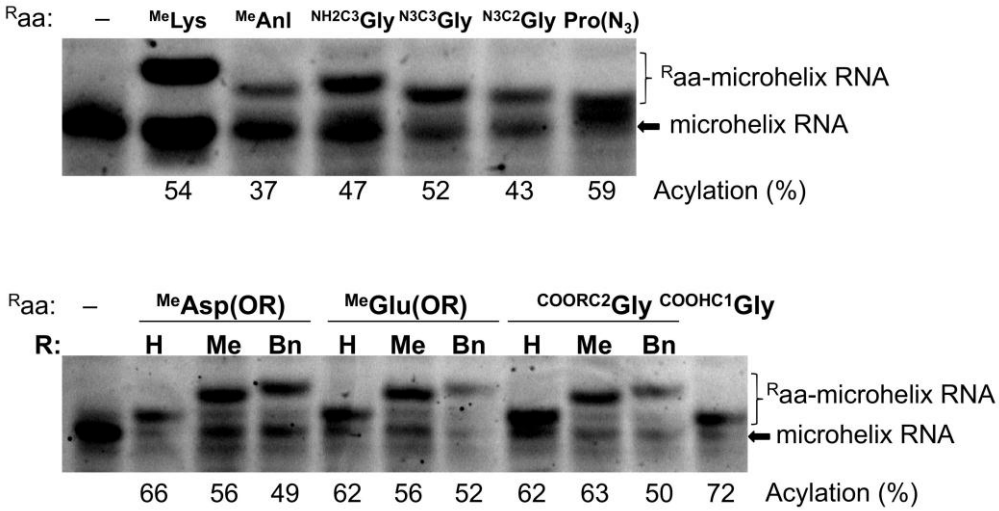


Fig. S2 Acid urea-PAGE of microhelix RNAs aminoacylated with *N*-alkyl amino acids using flexizyme. *N*-alkyl amino acids (^{R}aa) were charged onto a microhelix RNA using the dinitro-flexizyme (dFx) and the corresponding *N*-alkyl amino acid 3,5-dinitrobenzyl esters. The yields of the *N*-alkyl aminoacyl-(^{R}aa-)microhelix RNAs were calculated based on the fluorescence intensity of the ^{R}aa-microhelix RNAs (I) and the microhelix RNAs (II) using the expression (I) / [(I) + (II)].

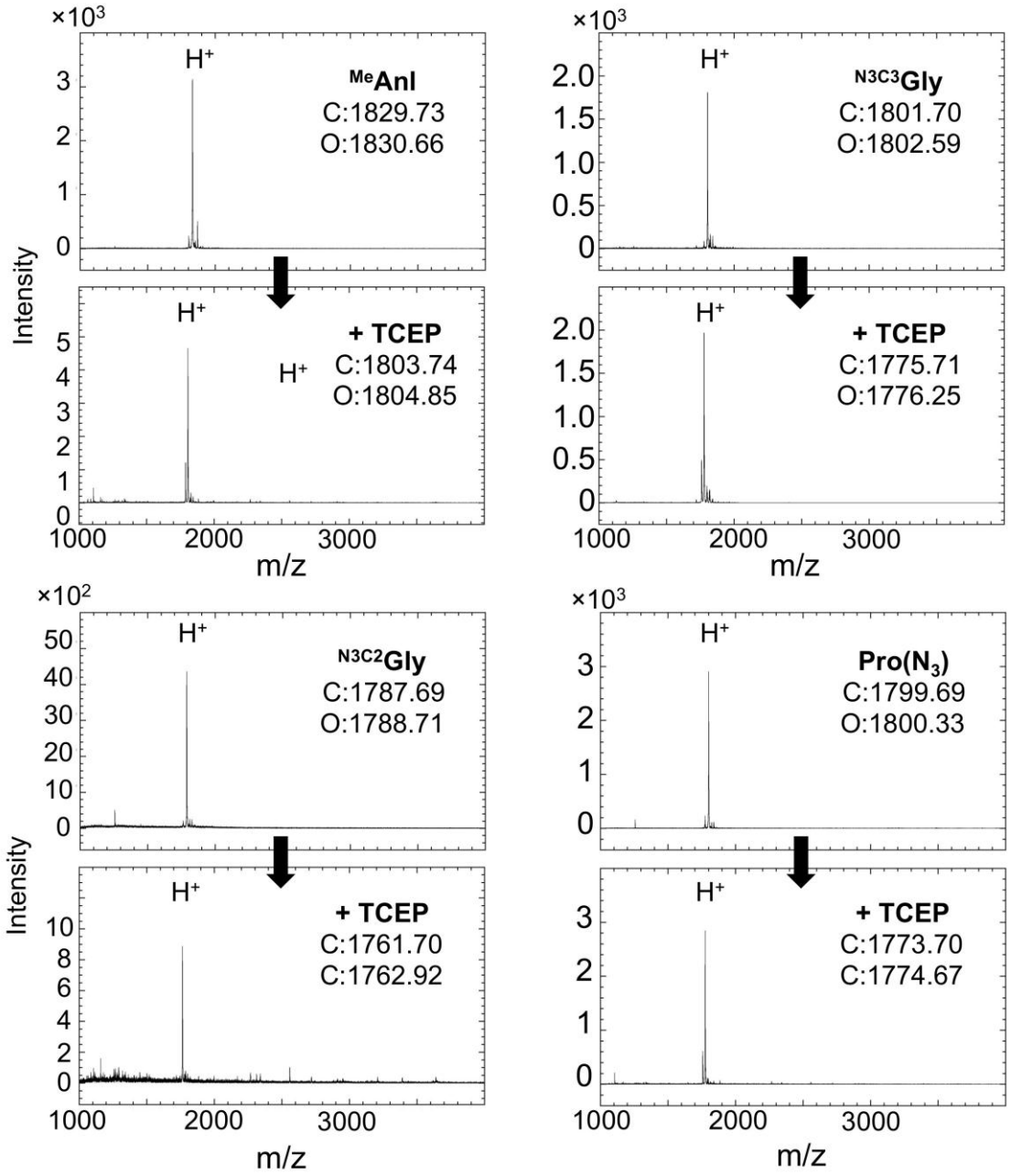


Fig. S3 Full MALDI-TOF mass spectra of the peptides containing the azido precursor of the *N*-alkyl amino acid and those after TCEP treatment shown in Fig. 2C. Calculated molecular mass of the desired peptides (C) and observed molecular mass of the major peak (O) for the singly charged species $[M+H]^+$ are shown in each spectrum.

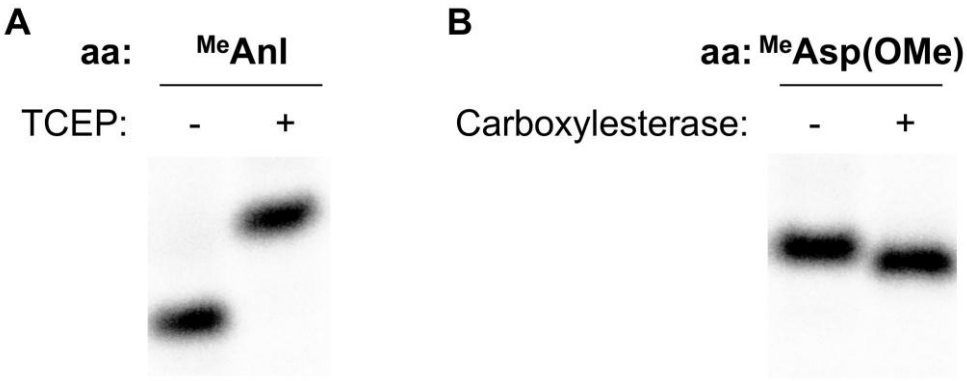


Fig. S4 Gel shift analysis of [^{14}C]-Asp-labeled peptides on Tricine-SDS-PAGE. (A) Tricine-SDS-PAGE of the ^{Me}Anl-containing peptide and that after TCEP-treatment. (B) Tricine-SDS-PAGE of the ^{Me}Asp(OMe)-containing peptide and that after esterase-treatment.

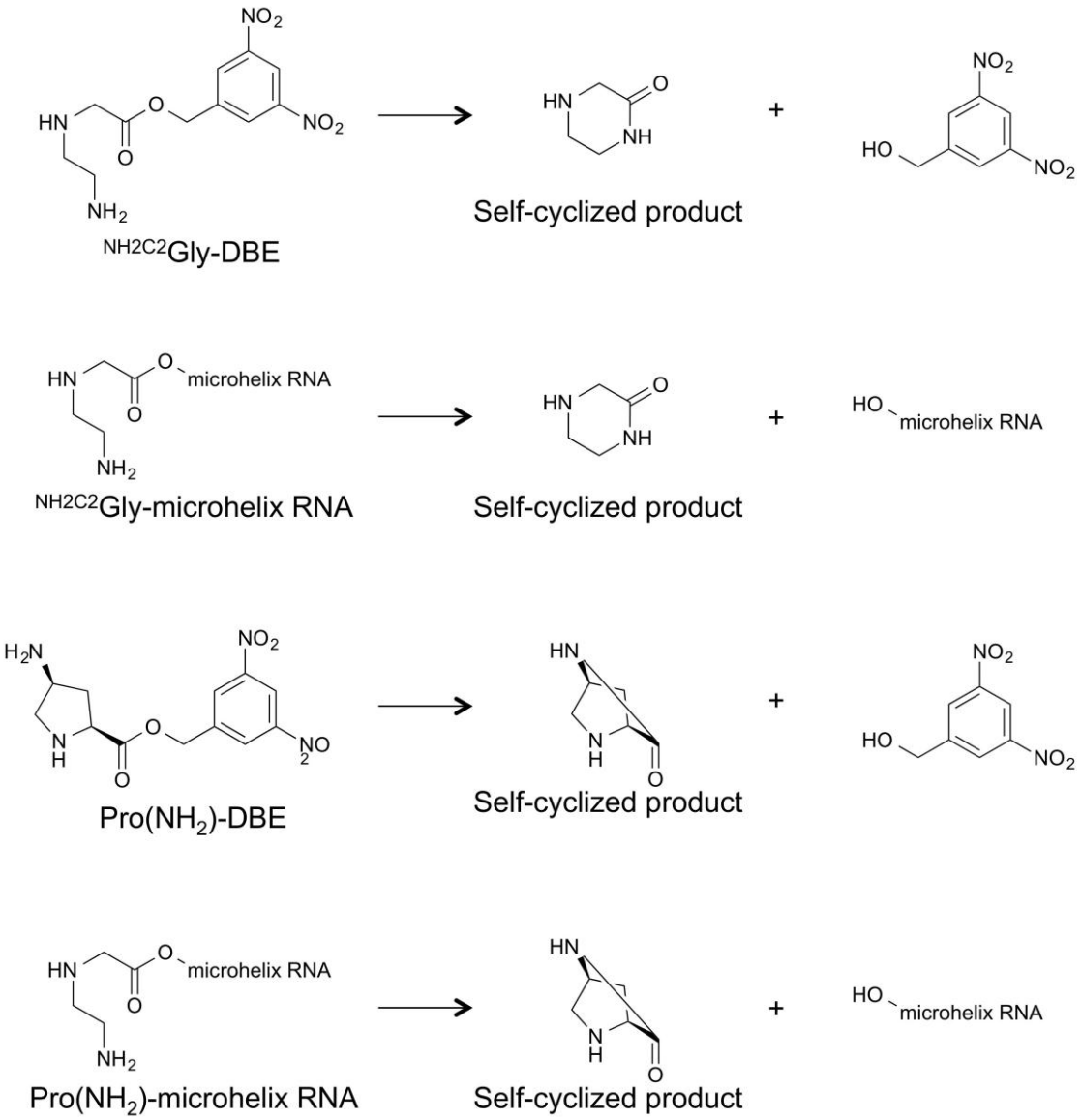


Fig. S5 Reaction mechanisms of self-cyclization of amino acid-DBEs and aminoacyl-microhelix RNAs.

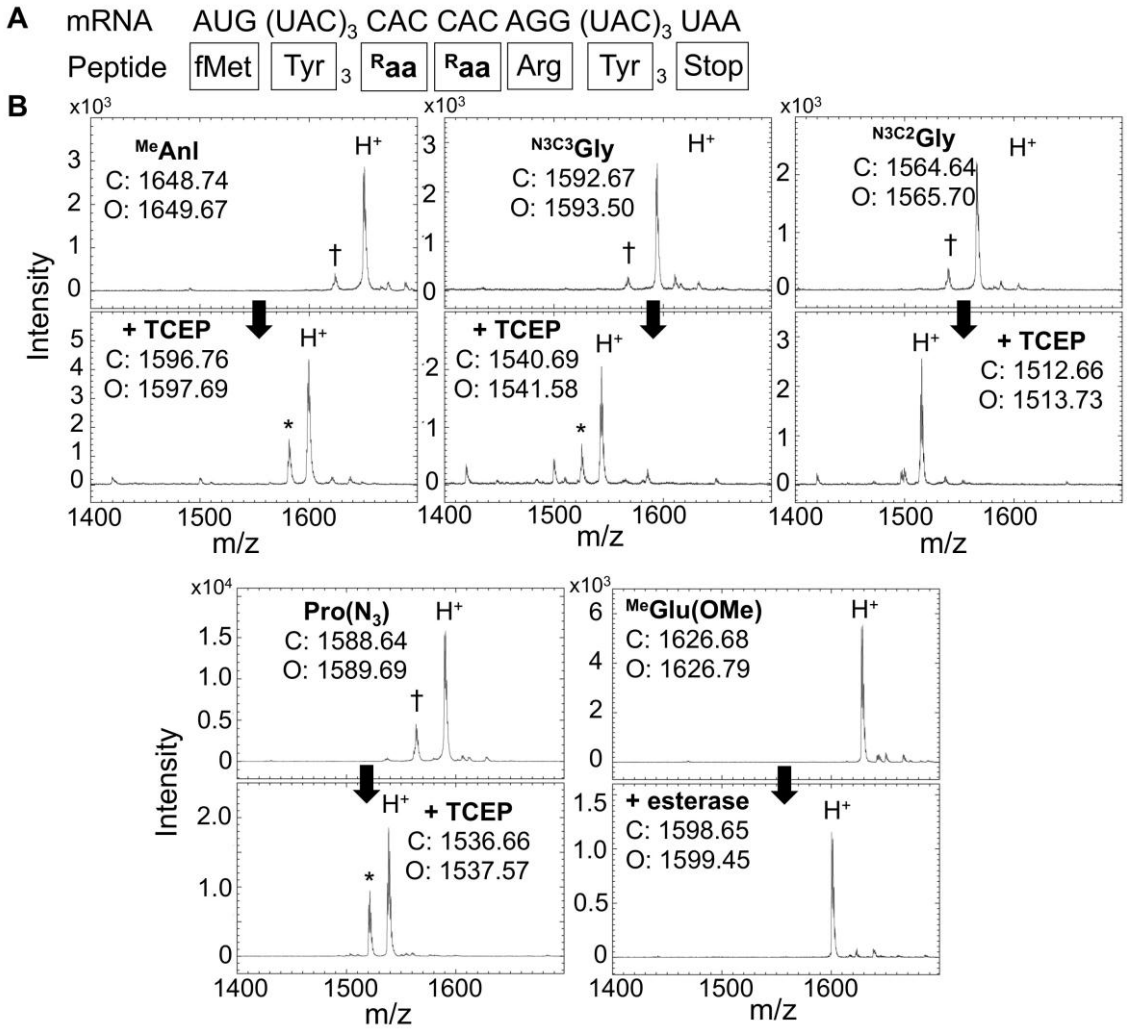
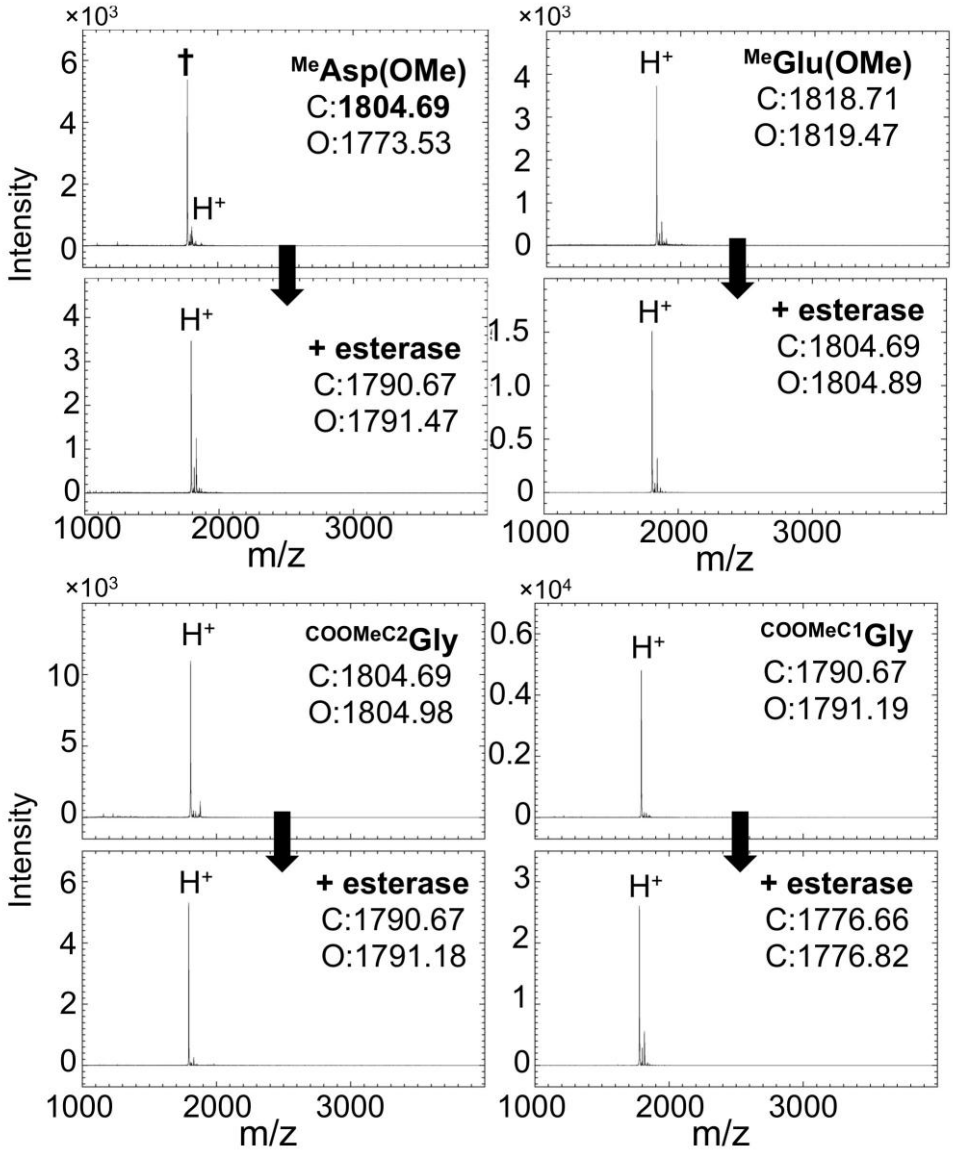


Fig. S6 MALDI-TOF mass of peptides for successive double incorporation of *N*-alkyl amino acids. (A) Sequences of mRNA and peptides encoded in the mRNA used for successive double incorporation of *N*-alkyl amino acids. *N*-alkyl amino acids were reassigned to the vacant CAC codon. (B) MALDI-TOF mass spectra of the translation products (first and third panels) and those after TCEP- or esterase-treatment (second and forth panels). Calculated molecular mass of the desired peptides (C) and observed molecular mass of the major peak (O) for the singly charged species $[M + H]^+$ are shown in each spectrum. H^+ corresponds to the protonated adduct ($[M + H]^+$) of the desired peptides. Minor peaks represented by "†" correspond to the peptides generated by the reduction of the azide to the amine by laser irradiation during MALDI-TOF-MS analysis[1]. We speculate that the minor peaks shown by "*" correspond to imido esters produced as a result of a similar mechanism to a previously reported one[2].



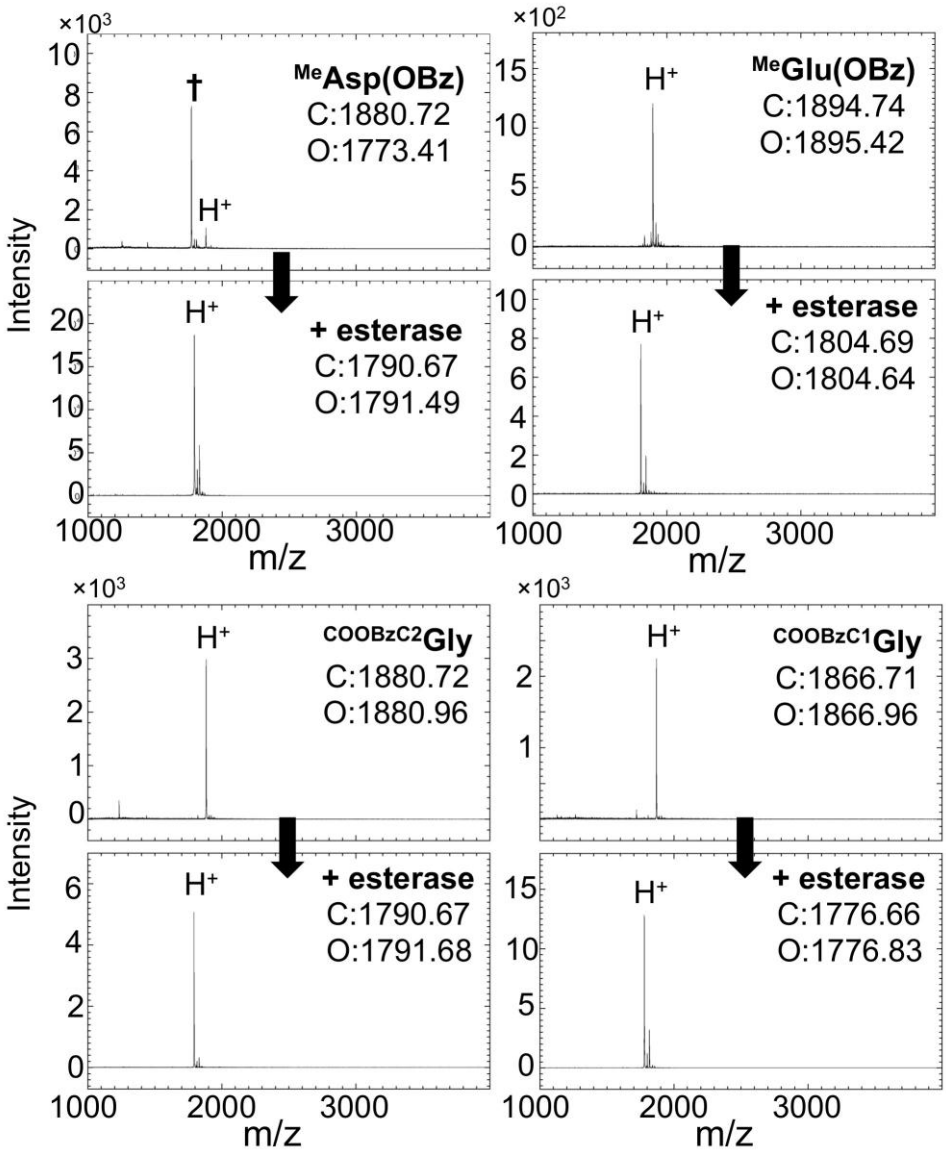


Fig. S7 Full MALDI-TOF mass spectra of the peptides containing the ester precursor of the *N*-alkyl amino acid and those after carboxylesterase treatment shown in Fig. 3B. Calculated molecular mass of the desired peptides (C) and observed molecular mass of the major peak (O) for the singly charged species $[M + H]^+$ are shown in each spectrum. The peaks represented by "†" correspond to the aspartimide-containing peptides.

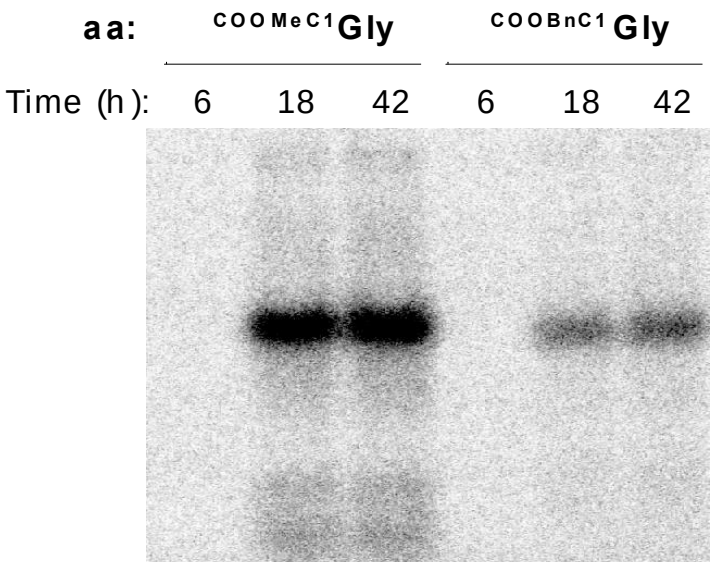


Fig. S8 Tricine-SDS-PAGE of the expressed peptides labeled with [^{14}C]-Asp, which were detected by autoradiography. The peptides were expressed in the presence of the ^{COOMeC1}Gly-tRNA$^{Asn\text{-}E2}_{GUG}$ or ^{COOMeC1}Gly-tRNA$^{Asn\text{-}E2}_{GUG}$ prepared using flexizyme in the designated aminoacylation time.

Table S1. Oligo DNAs used for preparing the template DNAs that encode peptides, for reverse transcription or for TRAP display. The sequences are written from left to right in the 5′ to 3′ direction. Oligo DNAs were purchased from Greiner Bio-One or BEX.

Names	Sequences
T7pEpsSD6MY3.F37	GGT TAACT TTAAC AAGGA GAAAA AC ATG TAC TAC TAC
eSD6MY3HFlag.R40	GTCGTCGTCCTTGTAGTC GTG GTAGTAGTACATGTTTTT
T7pEpsSD6.F40	GGCGT AATAC GACTC ACTAT AGGGT TAACT TTAAC AAGGA
Flaguaa.R33	CGAAGC TTA CTT GTC GTC GTC GTC CTT GTA GTC
eSD6MY3H2RY3.R40	TTAGTAGTAGTACCT GTGGTG GTAGTAGTACATGTTTTT
RY3uaa2.R18	TTATTAGTAGTAGTACCT
SD8M2-Y3H-G5S-4.F40	AGGTGATATTT ATG TAC TAC TAC CAT GGT GGA GGA GGA GG
G5S-4.an21.R41	CCCGCCTCCCGCCCCCCGTC CTA GCT ACC TCC TCC TCC ACC
T7SD8M2.F44	ATACTAATACGACTCACTATAGGATTAAGGAGGTGATATTTATG
G5S-4.R20	TAGCT ACCTC CTCCT CCACC
Puromycin-DNA linker	CCCGC CTCCC GCCCC CCGTC C- $(SPC18)_5$-CC-Puromycin

SPC18: 18-O-Dimethoxytritylhexaethyleneglycol,1-[(2-cyanoethyl)- (*N*,*N*-diisopropyl)]-phosphoramidite

Supplementary Methods

Synthesis of *N*-alkyl-amino acid 3,5-dinitrobenzyl esters

All *N*-alkyl amino acids, Boc-protected *N*-alkyl amino acids and Fmoc-protected *N*-alkyl amino acids were purchase from Watanabe Chemical, TCI, Chem-Impex or Apollo Scientific. Azide-containing *N*-alkyl amino amino acids were synthesized from the corresponding primary amine-containing *N*-alkyl amino amino acids using the same procedure as reported elsewhere[3]. All *N*-alkyl amino acids were converted to 3,5-dinitrobenzyl esters (DBEs) using the same procedure as previously described[4-6]. Methyl esterification of *N*-alkyl amino acid DBEs was performed with trimethylsilyldiazomethane. Benzyl esterification of *N*-alkyl amino acid DBEs was performed with benzyl bromide using the same procedure as 3,5-dinitrobenzyl esterification of amino acids[4].

Aminoacylation assay of microhelix RNA with *N*-alkyl amino acids by flexizyme

Microhelix RNA and dinitro-flexizyme (dFx) were prepared by the run-off transcription of appropriate templates as previously described[4, 5]. Aminoacylation efficiencies were determined using microhelix RNA. Reactions were generally carried out under the following conditions: 5 µL of 25 µM dFx, 25 µM microhelix RNA, and 5 mM *N*-alkyl amino acid DBE in 0.1 M Hepes-K buffer pH 7.5, 20 mM $MgCl_2$ and 20 % DMSO on ice. The procedure was as follows: 50 µM microhelix RNA in 0.2 M Hepes-K buffer pH 7.5 (2.5 µL) was heated at 95 ˚C for 1 min and cooled to room temperature over 5 min. $MgCl_2$ (100 mM, 1 µL) and dFx (250 µM, 0.5 µL) were added to the mixture. The reaction was initiated by addition of an *N*-alkyl amino acid DBE (25 mM, 1 µL in DMSO) and incubated on ice. The reaction was stopped by addition of 15 µL of the loading buffer (150 mM sodium acetate pH 5, 10 mM EDTA and 83% formamide). This sample was analyzed by 20 % denaturing acid PAGE (50 mM sodium acetate pH 5, 6 M urea). The RNA was stained with ethidium bromide and analyzed by Pharos FX (BIO-RAD).

Preparation of $tRNA^{Asn\text{-}E2}$ aminoacylated with *N*-alkyl amino acids by flexizyme

$tRNA^{Asn\text{-}E2}$ was prepared by the run-off transcription of appropriate templates as previously described[6, 7]. Aminoacylation of $tRNA^{Asn\text{-}E2}$ was generally carried out under the following conditions: 50 µL of 25 µM dFx, 25 µM $tRNA^{Asn\text{-}E2}$ and 5 mM *N*-alkyl amino acid DBE in 0.1 M Hepes-K buffer pH 7.5, 20 mM $MgCl_2$ and 20 % DMSO on ice. The procedure was as follows: 50 µM $tRNA^{Asn\text{-}E2}$ in 0.2 M Hepes-K buffer pH 7.5 (25 µL) was heated at 95 ˚C for 1 min and cooled to room temperature over 5 min. $MgCl_2$ (100 mM, 10 µL) and dFx (250 µM, 5 µL) were added to the mixture. The reaction was initiated by addition of a *N*-alkyl amino acid

DBE (25 mM, 10 µL in DMSO) and incubated on ice. The reaction was stopped by addition of 150 µL of 0.6 M sodium acetate pH 5. The RNA was recovered by ethanol precipitation, and rinsed with 70 % ethanol.

Preparation of template DNAs encoding peptides

Primers used to prepare template DNAs are listed in Table S1. A template DNA encoding peptide shown in Fig. 2A was prepared using forward primer (T7pEpsSD6MY3.F37) and reverse primer (eSD6MY3HFlag.R40) for primer extension and forward primer (T7pEpsSD6.F40) and reverse primer (Flaguaa.R33) for amplification. A template DNA encoding peptide shown in Fig. S6A was prepared using forward primer (T7pEpsSD6MY3.F37) and reverse primer (eSD6MY3H2RY3.R40) for primer extension and forward primer (T7pEpsSD6.F40) and reverse primer (RY3uaa2.R18) for amplification. A template DNA encoding peptide shown in Fig. 4B was prepared as follows. Forward primer (SD8M2-Y3H-G5S-4.F40) and reverse primer (G5S-4.an21.R41) were annealed and extend with Taq DNA polymerase (Genscript). The resulting dsDNA was then amplified with Taq DNA polymerase using forward primer (T7SD8M2.F44) and reverse primer (G5S-4.an21.R41).

Preparation of the reconstituted cell free translation system

The reconstituted translation system was prepared as described in the previous reports[5, 6, 8-10]. The concentration of the protein factors, tRNAs, small molecules, and ribosome in the translation reaction mixture were adjusted as described before[11]. 50 mM Hepes·KOH [pH 7.6], 12 mM $Mg(OAc)_2$, 100 mM potassium acetate, 2 mM spermidine, 1 mM DTT, 20 mM creatine phosphate (Roche), 2 mM ATP, 2 mM GTP, 1 mM CTP, 1 mM UTP, 0.1 mM 10-formyl-5,6,7,8-tetrahydrofolic acid, 1.5 mg/ml *E. coli* total tRNAs (Roche), 0.6 µM MTF, 2.7 µM IF1, 0.4 µM IF2, 1.5 µM IF3, 0.26 µM EF-G, 10 µM EF-Tu, 10 µM EF-Ts, 0.25 µM RF2, 0.17 µM RF3, 0.5 µM RRF, 0.1 µM T7 RNA polymerase, 0.1 µM nucleotide-diphosphate kinase, 4 µg/ml creatine kinase (Roche), 3 µg/ml myokinase (Sigma), 0.1µM pyrophosphatase, and 1.2 µM ribosome.

Preparation of the TRAP system

The TRAP system was prepared with the above translation system containing 2.5 µM puromycin-DNA linker (BEX, Japan) and 1 µM T7 RNA polymerase according to the procedure described elsewhere[11, 12].

Preparation of *N*-biotinyl-Phe-tRNA$^{fMet}_{CAU}$

N-biotinyl-Phe-tRNA$^{fMet}_{CAU}$ was prepared as previously described[12]. Aminoacylation of tRNA$^{fMet}_{CAU}$ were carried out under the following conditions: 50 µL of 25 µM eFx, 25 µM tRNA$^{fMet}_{CAU}$ and 5 mM *N*-biotinyl-Phe-CME in 0.1 M Hepes-K buffer pH 8, 600 mM $MgCl_2$ and 20 % DMSO on ice. The procedure was as follows: 50 µM tRNA$^{fMet}_{CAU}$ in 0.2 M Hepes-K buffer pH 7.5 (25 µL) was heated at 95 ˚C for 1 min and cooled to room temperature over 5 min. $MgCl_2$ (3 M, 10 µL) and eFx (250 µM, 5 µL) were added to the mixture. The reaction was initiated by addition of a *N*-biotinyl-Phe-CME (25 mM, 10 µL in DMSO) and incubated on ice. The reaction was stopped by addition of 150 µL of 0.6 M sodium acetate pH 5. The RNA was recovered by ethanol precipitation, and rinsed twice with 70 % ethanol containing 0.1 M sodium acetate pH 5, and once with 70 % ethanol.

Ribosomal synthesis of peptides containing successive double *N*-alkyl amino acids

Translation reaction mixture containing 0.04 µM DNA template, 0.5 mM each Met, Tyr, Arg, 0.03 µM MetRS, 0.02 µM TyrRS, 0.03 µM ArgRS, and 100 µM *N*-alkyl aminoacyl-tRNA$^{Asn\text{-}E2}_{GUG}$ was incubated for 60 min at 37˚C. For MALDI-TOF mass analysis, the translation product was desalted with C-TIP (Nikkyo Technos), eluted with 80% acetonitrile, 0.5% acetic acid saturated with CHCA and analyzed by autoflex II (BRUKER DALTONICS) operated in the linear positive mode.

References

1. Y. Sako, J. Morimoto, H. Murakami and H. Suga, *J. Am. Chem. Soc.*, 2008, **130**, 7232-7234.
2. J. W. Back, O. David, G. Kramer, G. Masson, P. T. Kasper, L. J. de Koning, L. de Jong, J. H. van Maarseveen and C. G. de Koster, *Angew. Chem. Int. Ed.*, 2005, **44**, 7946-7950.
3. A. J. Link, M. K. Vink and D. A. Tirrell, *Nat. Protoc.*, 2007, **2**, 1879-1883.
4. H. Murakami, A. Ohta, H. Ashigai and H. Suga, *Nat. Methods*, 2006, **3**, 357-359.
5. T. Kawakami, H. Murakami and H. Suga, *Chem. Biol.*, 2008, **15**, 32-42.
6. T. Kawakami, H. Murakami and H. Suga, *J. Am. Chem. Soc.*, 2008, **130**, 16861-16863.
7. A. Ohta, H. Murakami, E. Higashimura and H. Suga, *Chem. Biol.*, 2007, **14**, 1315-1322.
8. Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa and T. Ueda, *Nat. Biotechnol.*, 2001, **19**, 751-755.
9. H. Ohashi, Y. Shimizu, B. W. Ying and T. Ueda, *Biochem. Biophys. Res. Commun.*, 2007, **352**, 270-276.

10. T. Kawakami, A. Ohta, M. Ohuchi, H. Ashigai, H. Murakami and H. Suga, *Nat. Chem. Biol.*, 2009, **5**, 888-890.
11. T. Kawakami, T. Ishizawa, T. Fujino, P. C. Reid, H. Suga and H. Murakami, *ACS Chem. Biol.*, 2013, **8**, 1205-1214.
12. T. Ishizawa, T. Kawakami, P. C. Reid and H. Murakami, *J. Am. Chem. Soc.*, 2013, **135**, 5433-5440.