Table of contents

Reagents

Unless otherwise noted, all chemical reagents were obtained from commercial sources and used without further purification. Creatine kinase was purchased from Roche. Myokinase was purchased from SIGMA. Unless otherwise noted, all commercially available enzymes and buffers were purchased from Nacalai Tesque. eFx, $tRNA^{fMet}_{CAU}$, $tRNA^{AsnE2}_{CCA}$, ^{ClAc}Phe cyanomethyl ester, and ^{HO}Phe cyanomethyl ester were synthesized with the same procedure as previously reported.[1–4]

DNA oligomers

All oligonucleotides used in this study were purchased from OPERON biotechnology (Japan) and listed below.

O1: 5'-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG-3'

O2: 5'-GGCGTAATACGACTCACTATAG-3'

O3: 5'-CGAAGCTTACTTGTCGTCGTCGTCCTTGTAGTC-3'

O4: 5'-AGGTCAGCGGGCCAAAATGGCTATAGCTGGTCATATGTATATCTCCTTC-3'

O5: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTCACCCAGGTCAGCGGGCCAAAA-3'

O6: 5'-AGGTCAGCGGGCCAAAATGGCTATAGGTGCACATATGTATATCTCCTTC-3'

O7: 5'-AGGTCAGCGGGCCAAAATGGCTATAGCAGGTCATATGTATATCTCCTTC-3'

O8: 5'-AGGTCAGCGGGCCAAAATGGCAGCTATAGGTCATATGTATATCTCCTTC-3'

O9: 5'-AGGTCAGCGGGCCGCAAAAATGGCTATAGGTCATATGTATATCTCCTTC-3'

O10: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTCACCCAGGTCAGCGGGCCGCAA-3'

O11: 5'-AGGTCAGGCACGGGCCAAAATGGCTATAGGTCATATGTATATCTCCTTC-3'

O12: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTCACCCAGGTCAGGCACGGGCCA-3'

O13: 5'-AGCAGGTCAGCGGGCCAAAATGGCTATAGGTCATATGTATATCTCCTTC-3'

O14: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTCACCCAGCAGGTCAGCGGGCCA-3'

O15: 5'-CCCAGGTCAGCGGGCCAAAATGGCTATAGGTCATATGTATATCTCCTTC-3'

O16: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTGCACACCCAGGTCAGCGGGCCA-3'

O17: 5'-AGGTGCACGGGCCAAAATGGCTATAGGTGCACATATGTATATCTCCTTC-3'

O18: 5'-GTCGTCCTTGTAGTCGCACTGCGGTTTCACCCAGGTGCACGGGCCAAAA-3'

O19: 5'-AGGTCAGCGGGCCAAAATGGCAGCTATAGCACATATGTATATCTCCTTC-3'

O20: 5'-GTCGTCCTTGTAGTCCTGCGGTTTGCACACCCAGGTCAGCGGGCCAAAA-3'

O21: 5'-CAGGTTGTTAATGCACTGCAGGCTGCACATATGTATATCTCCTTC-3'

O22: 5'-GTCGTCCTTGTAGTCGCCACCACCCGGCCAGCACAGGTTGTTAATGCAC-3'

O23: 5'-ACAGGAGTCACCCGCCAACACAAGCACATATGTATATCTCCTTC-3'

O24: 5'-GTCGTCCTTGTAGTCGCAAATAAACGAACTAGGAGTCACCCGCC-3'

O25: 5'-GCCACCGCAGCCGCACATATGTATATCTCCTTC-3'

O26: 5'-GTCGTCCTTGTAGTCGCAGCCACCGCAGCCGCA-3'

O27: 5'-CGCTTTTAAAGCACACTTCGCACATATGTATATCTCCTTC-3'

O28: 5'-GTCGTCCTTGTAGTCGCACGCGCTTTTAAAGCACACTTC-3'

O29: 5'-GCACGCGCTCGCCCAAATGCACATATGTATATCTCCTTC-3'

O30: 5'-GTCGTCCTTGTAGTCGCAAATTTTGCACGCGCTCGCCCAAATG-3'

O31: 5'-CCCAGGTCAGCGGGCCAAAATGGCTGCAGGTCATATGTATATCTCCTTC-3'

O32: 5'-GAGTCACAAGATGACAAATCAGAGTACAATGCATATGTATATCTCCTTC-3'

O33: 5'-GTCGTCCTTGTAGTCGCACACAAAAGTAGCCTGAGTCACAAGATGACAA-3'

Preparation of mDNA for peptides synthesis

For the preparation of mDNA1–17, O1 and O4, O6, O7, O8, O9, O11, O13, O15, O17, O19, O21, O23, O25, O27. O29, O31, or O32 were annealed, respectively, and extended by *Taq* DNA polymerase. The resulting dsDNAs were amplified using O2 as a forward primer and O5, O5, O5, O5, O10, O12, O14, O16, O18, O20, O22, O24, O26, O28, O30, O16, or O33 as a reverse primer for mDNA1–17, respectively, and then these products were further amplified using O2 and O3. Prepared DNA templates were purified by phenol/chloroform extraction and ethanol precipitation.

mDNA1:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCAGCTATAGCCAT

TTTGGCCCGCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA2:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCACCTATAGCCAT

TTTGGCCCGCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA3:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTGCTATAGCCAT

TTTGGCCCGCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA4:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTATAGCTGCCAT

TTTGGCCCGCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA5:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTATAGCCATTTTT

GCGGCCCGCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA6:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTATAGCCATTTT

GGCCCGTGCCTGACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA7:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTATAGCCATTTT

GGCCCGCTGACCTGCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA8:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTATAGCCATTTT

GGCCCGCTGACCTGGGTGTGCAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA9:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCACCTATAGCCAT

TTTGGCCCGTGCACCTGGGTGAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA10:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCTATAGCTGCCATT

TTGGCCCGCTGACCTGGGTGTGCAAACCGCAGGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA11:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCAGCCTGCAGTGC

ATTAACAACCTGTGCTGGCCGGGTGGTGGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA12:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCTTGTGTTGGCGG

GTGACTCCTAGTTCGTTTATTTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA13:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGGCTGCGGTGGC

TGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA14:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGAAGTGTGCTTT

AAAAGCGCGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA15:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCATTTGGGCGAGC

GCGTGCAAAATTTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA16:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCTGCAGCCATTTT

GGCCCGCTGACCTGGGTGTGCAAACCGCAGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

mDNA17:

5'-GGCGTAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGCATTGTACTCTGATTT

GTCATCTTGTGACTCAGGCTACTTTTGTGTGCGACTACAAGGACGACGACGACAAGTAAGCTTCG-3'

Preparation of acyl-tRNAs

Acyl-tRNAs were prepared by the following procedure. 40 μM tRNA and 40 μM eFx in 6 μL of 80 mM HEPES-K buffer pH 7.5 was incubated at 95°C for 2 min and cooled to 25°C for 5 min. 2 μL of 3 M $MgCl_2$ was added and the mixture was incubated at 25°C for 5 min. The reaction was initiated by addition of 2 μL of 25 mM amino acid cyanomethyl ester substrate in DMSO and incubated on ice for 2 h. After acylation, the reaction was stopped by addition of 40 μL of 0.3 M sodium acetate pH 5.2, and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate pH 5.2, and once with 70% ethanol. The acyl-tRNAs were dissolved in 1 μL of 1 mM sodium acetate pH 5.2 just before adding to translation mixture. $tRNA^{fMet}_{CAU}$[2] and ^{ClAc}Phe cyanomethyl ester were used for the synthesis of ^{ClAc}Phe-$tRNA^{fMet}_{CAU}$. $tRNA^{AsnE2}_{CCA}$[3] and ^{HO}Phe cyanomethyl ester were used for the synthesis of ^{HO}Phe-$tRNA^{AsnE2}_{CCA}$.

Ribosomal synthesis of peptides

A reconstituted cell free translation system containing all necessary components for translation except for Met (and Trp) was used in this study. The translation system was prepared as previously reported.[5] The translation mixtures contained final concentrations of 50 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 2 mM GTP, 2 mM ATP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate (Roche), 12 mM $Mg(OAc)_2$, 2 mM spermidine, 2 mM DTT, 100 μM 10-formyl-5,6,7,8-tetrahydrofolic acid, 1.5 mg/mL *E. coli* total tRNA (Roche), 1.2 μM ribosome, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 10 μM EF-Tu, 0.66 μM EF-Ts, 0.26 μM EF-G, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 0.6 μM MTF, 4 μg/mL creatine kinase (Roche), 3 μg/mL myokinase (Sigma), 0.1 μM pyrophosphatase (Sigma), 0.1 μM nucleotide-diphosphatase kinase, 0.1 μM T7 RNA polymerase, 0.73 μM AlaRS, 0.03 μM ArgRS, 0.38 μM AsnRS, 0.13 μM AspRS, 0.02 μM CysRS, 0.06 μM GlnRS, 0.23 μM GluRS, 0.09 μM GlyRS, 0.02 μM HisRS, 0.40 μM IleRS, 0.04 μM LeuRS, 0.11 μM LysRS, 0.03 μM MetRS, 0.68 μM PheRS, 0.16 μM ProRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.03 μM TrpRS, 0.02 μM TyrRS, 0.02 μM ValRS, the proteinogenic amino acids except for Met (each 200 μM), and 50 μM ^{ClAc}Phe-$tRNA^{fMet}_{CAU}$. The translation mixture was incubated at 37°C for 30 min. The translation reaction was usually performed in a 5 μL-scale.

Mass spectrometry of peptides

The translation products from mDNA1–17 were purified with FLAG-M2 agarose (Sigma). After the resin was washed with 1×TBS buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl), the peptides were eluted with 10 μL of 0.2% TFA. The purified peptides were desalted with C-TIP (Nikkyo Technos), and eluted with 1 μL of an 80% acetonitrile, 0.5% acetic acid solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid (Bruker Daltonics).

MALDI-MS measurement was performed using autoflex II TOF/TOF (Bruker Daltonics) under the linear/positive or reflection/positive mode and externally calibrated with peptide calibration standard II (Bruker Daltonics).

General procedure for hydrolysis of ester bonds in peptides

For the expression of ester bond-containing peptide, the translation mixture did not include Trp, but contained 50 μM ^{HO}Phe-tRNA$^{EnAsn}_{CCA}$. In this case, the translation mixture was incubated for 2 h, and the translation reaction was performed in a 2.5 μL-scale. After the FLAG purification, 0.56 μL of 1 M sodium carbonate buffer pH 10.0 was added to 5 μL of the solution, and the mixture was incubated at 37°C for 30 min for hydrolysis of the ester bond. After adding 5 μL of 2% TFA, the mixture was desalted with C-TIP and subjected to MALDI-MS measurement.

General procedure for reduction of disulfide bonds in peptides

For the expression of oxidative peptides, the translation mixture was incubated for 3 h, and the translation was performed in a 2.5 μL-scale. After the FLAG purification, 0.56 μL of 1 M Tris-HCl pH 7.5 and 0.50 μL of 200 mM tris(2-carboxyethyl)phosphine hydrochloride was added to 5 μL of the solution, and the mixture was incubated at 37°C for 10 min for reduction of the disulfide bond. After adding 5 μL of 2% TFA, the reduction product was desalted with C-TIP and subjected to MALDI-MS measurement.

Supporting Figures

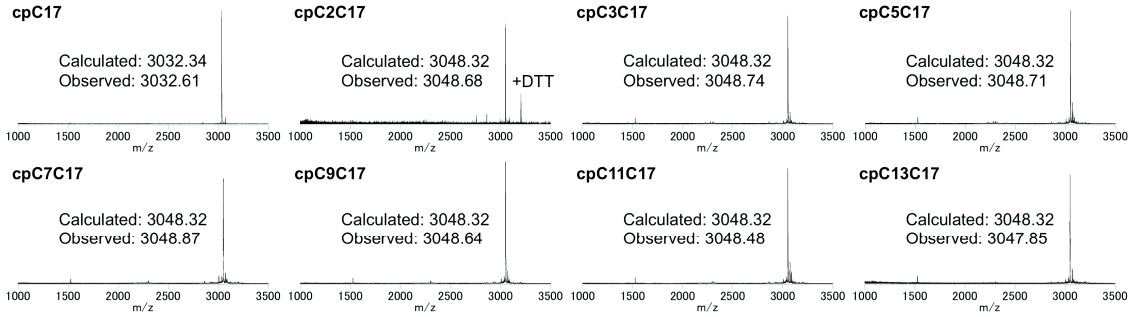

Fig. S1 MALDI-TOF spectra of peptides expressed in this study (cpC17–cpC13C17). In the case of cpC2C17, peaks corresponding to a mixture of two minor products were observed in the region of 3200–3208 m/z. These minor products were attributed to a DTT adduct of ^{u}C2 of the major product and a DTT adduct of N-terminus (DTT was present in the translation solution). The latter product could be generated by a remaining unreacted fraction of linear pC2C17 was oxidatively linked to DTT via a disulfide bond, yielding intramolecular addition to the N-terminal ClAc group, although the mechanism is not proved.

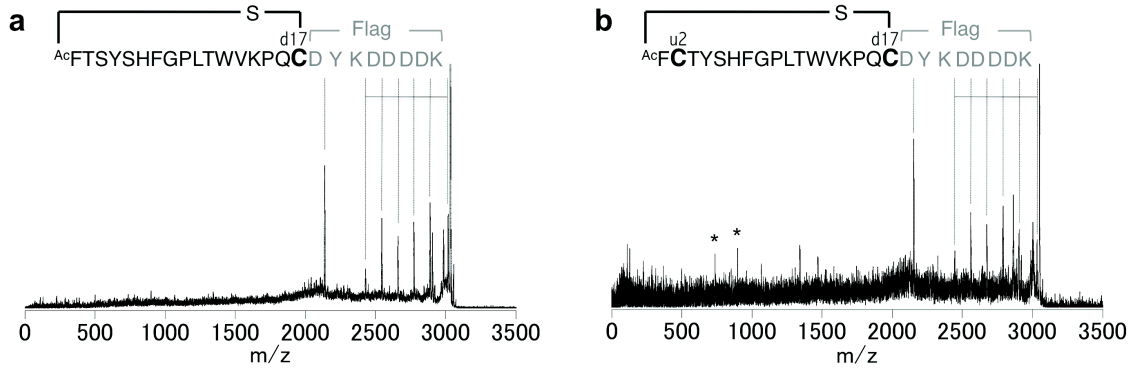

Fig. S2 MALDI-TOF/TOF spectra of (a) cpC17 and (b) cpC2C17. Assigned b-ion peaks are shown in the figure. Peaks labeled with asterisks are assigned as y-ion peaks.

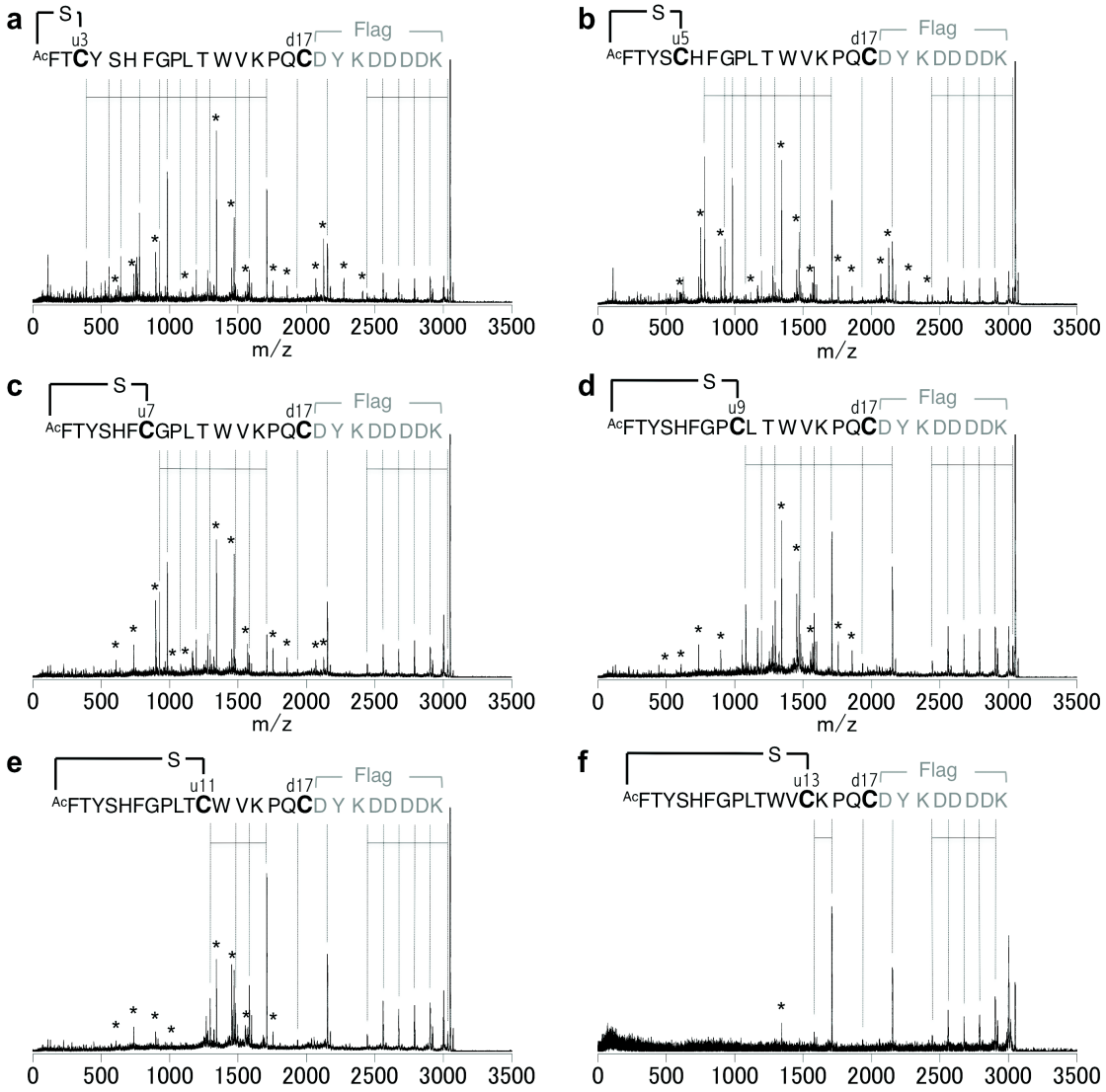

Fig. S3 MALDI-TOF/TOF spectra of (a) cpC3C17, (b) cpC5C17, (c) cpC7C17, (d) cpC9C17, (e) cpC11C17, and (f) cpC13C17. Assigned b-ion peaks are shown in the figure. Peaks labeled with asterisks are assigned as y-ion peaks.

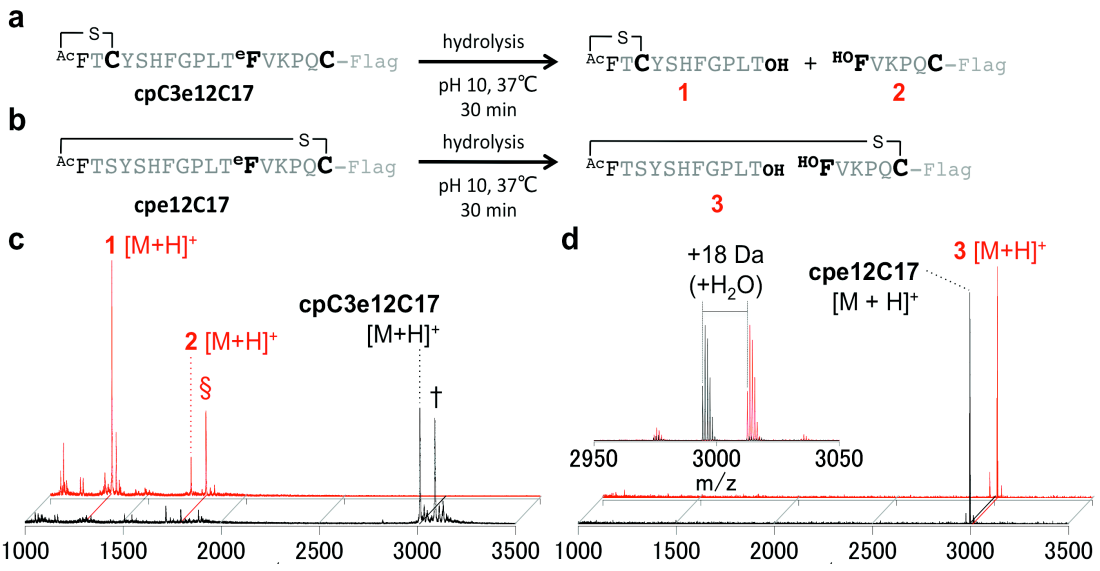

Fig. S4 Validation of thioether macrocyclic peptides by the incorporation of phenyllactic acid. (**a**) Expression of cpC3e12C17 where W12 was replaced with ^{HO}F in the parental peptide, cpC3C17, yielded the desired full-length peptides with an ester bond at the 12th position. Upon cleavage of the ester bond by alkaline hydrolysis, cpC3e12C17 would be fragmented to yield **1** and **2**. (**b**) Expression of cpe12C17, where the same substitution as cpC3e12C17 was performed. Cleavage of the ester bond by alkaline hydrolysis should yield a single hydrolyzed product (**3**). (**c**) MALDI-TOF analysis of cpC3e12C17 before and after alkaline hydrolysis. cpC3e12C17 $[M+H]^+$, Cal. 3010.29, Obs. 3011.38; **1** $[M+H]^+$, Cal. 1312.57, Obs. 1312.23; **2** $[M+H]^+$, Cal. 1716.74, Obs. 1716.53. † and § indicate the peaks corresponding to the β-mercaptoethanol adduct of cpC3e12C17 and **2**, respectively, via a disulfide bond with the free sulfhydryl group of ^{d}C. (**d**) MALDI-TOF analysis of cpe12C17 before and after alkaline hydrolysis. Calculated (Cal.) and observed (Obs.) mass values of each peak are as follows: cpe12C17 $[M+H]^+$, Cal. 2994.31, Obs. 2994.34; **3** $[M+H]^+$, Cal. 3012.32, Obs. 3012.59. The inset figure shows an expanded 2950–3050 m/z area of the superimposed spectra of cpe12C17 and **3**.

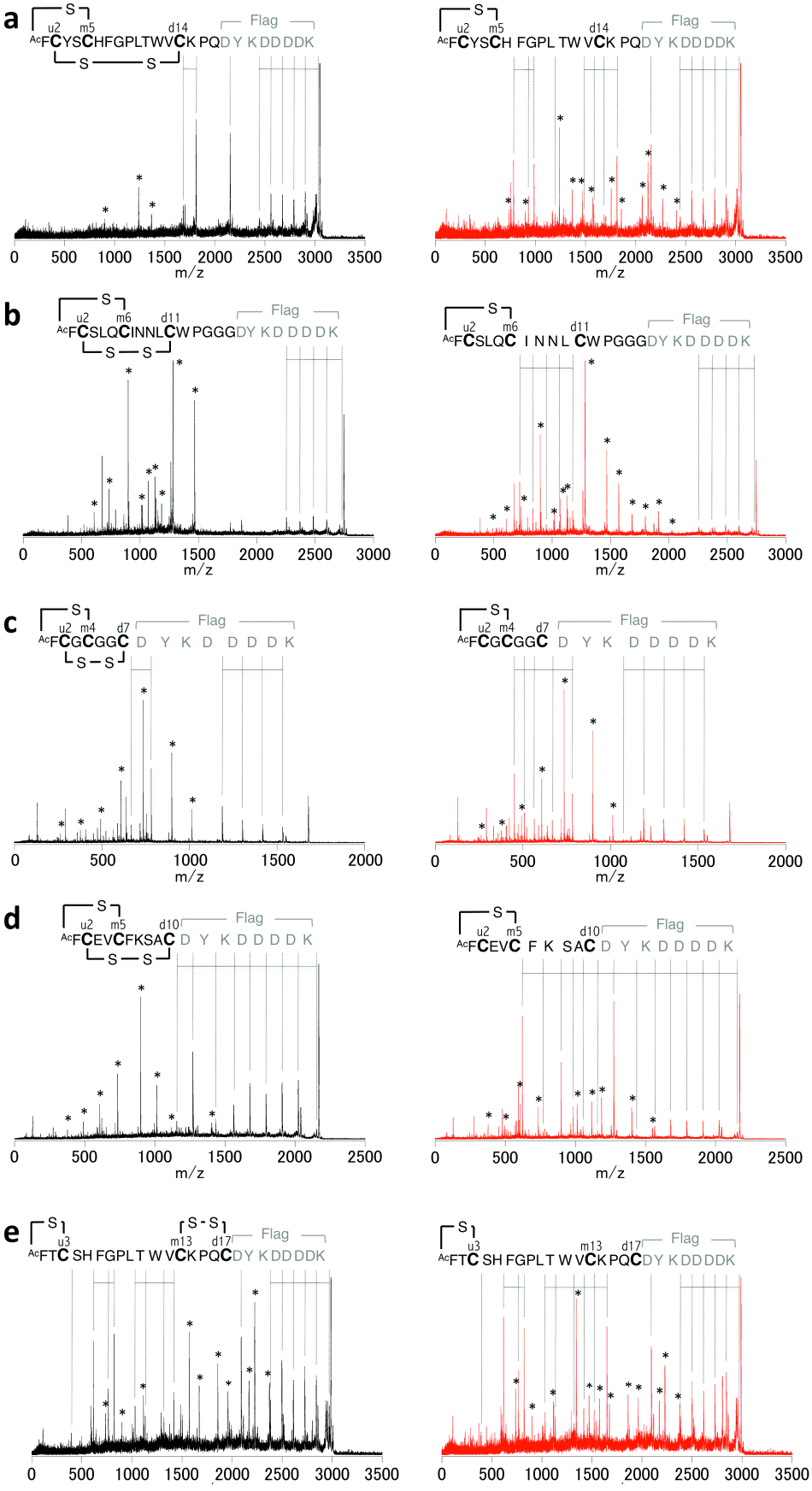
a
b
c
d
e
Flag
m/z

Fig. S5 MALDI-TOF/TOF spectra of (a) cpC2C5C14, (b) cpC2C6C11, (c) cpC2C4C7, (d) cpC2C5C10, and (e) cpC3C13C17. Mild air-oxidation of the each expressed peptide yielded the bicyclic structure that was consistent with the molecular mass containing a thioether bond and a disulfide bond (spectra of peptide-oxi shown in black). Upon the treatment with TCEP, the molecular mass of each peptide-oxi increased by 2 Da, suggesting that the disulfide bond was reduced to yield monocyclic peptide (spectra peptide-red shown in red). Assigned b-ion peaks are shown in the figure. Peaks labeled with asterisks are assigned as y-ion peaks.

References

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[4] Y. Goto, T. Katoh, H. Suga, *Protoc. Exchange* **2011**, doi:10.1038/protex.2011.209.

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