Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Ribosomal incorporation of cyclic β-amino acids into peptides using *in vitro* translation

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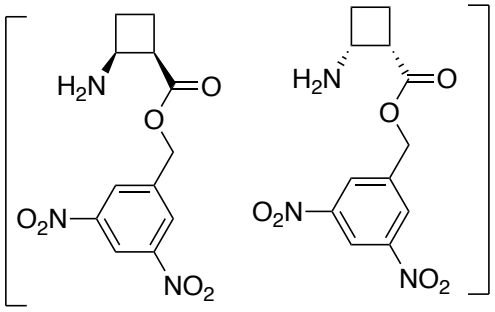
Materials and Methods

All reagents and solvents were commercial grade and purified prior to use when necessary. Dichloromethane was dried by passage through a column of activated alumina as described by Grubbs.[1]

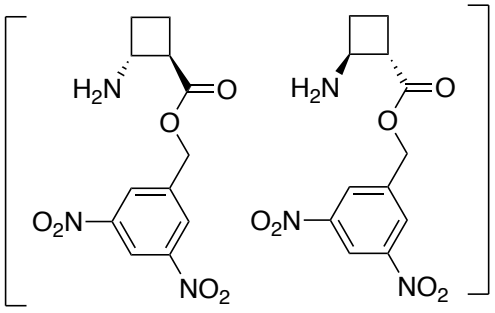
The substrates containing a DNB and CME ester were prepared as previously described[2]. Thin layer chromatography (TLC) was performed using glass-backed silica gel (250 μm) plates. UV light and/or the use of $KMnO_4$ were used to visualize products. Flash chromatography was performed on a Biotage Isolera One automated purification system or on a silica column.

Nuclear magnetic resonance spectra (NMR) were acquired on a Bruker Advance III-500 (500 MHz) instrument and processed by TopSpin. Chemical shifts are measured relative to residual solvent peaks as an internal standard set to δ 2.50 and δ 39.5 (DMSO-d_6). Mass spectra were recorded on a Bruker AmaZon SL (ESI) and the data were processed with Compass DataAnalysis 4.2 software (Bruker).

Characterization of substrates

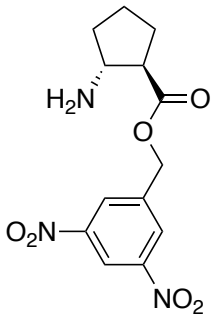


***cis*-3,5-dinitrobenzyl-2-aminocyclobutane-1-carboxylate (1a).** Prepared using cis-2-((tert-butoxycarbonyl)amino)cyclobutane-1-carboxylic acid (71 mg, 0.33 mmol), triethylamine (70 μL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) in dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (s, 1H), 8.74 (s, 2H), 5.42 (dd, J = 14.6 Hz, 2H), 3.95 (s, 1H), 3.61 (br,1H), 2.30 (br, 2H), 2.14 (br, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 171.5, 148.5 (2C), 140.6, 129.1 (2C), 118.7, 64.7, 45.9, 40.7. 25.4, 20.2; MS (ESI): Mass calcd for $C_{12}H_{13}N_3O_6$ $[M+H]^+$ 296.08, found 296.07.

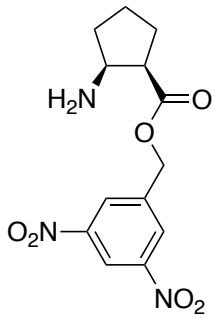


***trans*-3,5-dinitrobenzyl-2-aminocyclobutane-1-carboxylate (1b).** Prepared using trans-2-((tert-butoxycarbonyl)amino)cyclobutane-1-carboxylic acid (71 mg, 0.33 mmol), triethylamine (70 μL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) in dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.81 (t, J = 2.1 Hz, 1H), 8.70 (s, J = 2.1 Hz, 2H), 5.39 (dd, J = 17.5, 13.5 Hz, 2H), 3.87 (br, 1H), 3.65 (m, 2H), 2.80 (m, 3H), 1.95 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 171.7, 148.5 (2C), 140.8,

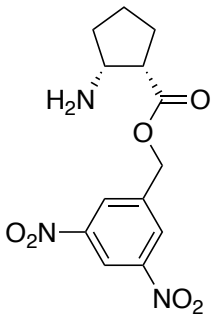
128.8 (2C), 118.6, 64.6, 46.5, 42.6. 23.9, 19.6; MS (ESI): Mass calcd for $C_{12}H_{13}N_3O_6$ $[M+H]^+$ 296.08, found 296.05.



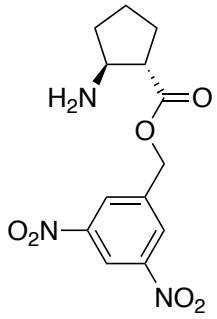
3,5-dinitrobenzyl (1R,2R)-2-aminocyclopentane-1-carboxylate (2a). Prepared using (1R,2R)-2-((tert-butoxycarbonyl)amino)cyclopentane-1-carboxylic acid (102 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.81 (s, 1H), 8.71 (d, J = 2.0 Hz, 2H), 5.40 (dd, J = 21.5, 13.5 Hz, 2H), 3.02 (m, 1H), 2.14 (m, 1H), 2.05 (m, 1H), 1.83 (t, J = 10.5 Hz, 2H), 1.75 (m, 2H).^{13}C NMR (125 MHz, DMSO-d_6) ppm 173.1, 148.5 (2C), 140.8, 128.7 (2C), 118.6, 64.6, 53.8, 48.2, 31.2, 29.6, 23.6; MS (ESI): Mass calcd for $C_{13}H_{15}N_3O_6$ $[M+H]^+$ 310.09, found 310.09.



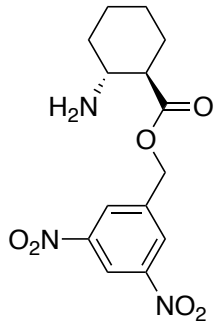
3,5-dinitrobenzyl (1R,2S)-2-aminocyclopentane-1-carboxylate (2b). Prepared using (1R,2S)-2-((tert-butoxycarbonyl)amino)cyclopentane-1-carboxylic acid (102 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, J = 2.0 Hz, 1H), 8.74 (d, J = 2.0 Hz, 2H), 5.40 (m, 2H), 3.73 (br, 1H), 3.20 (dd, J = 15.0, 8.5 Hz, 1H), 2.00 (m, 3H), 1.82 (m, 2H), 1.73 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 171.9, 148.5 (2C), 140.7, 128.9 (2C), 118.6, 64.7, 52.8, 46.3, 30.4, 26.7, 21.6; MS (ESI): Mass calcd for $C_{13}H_{15}N_3O_6$ $[M+H]^+$ 310.09, found 310.08.



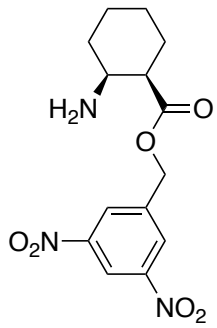
3,5-dinitrobenzyl (1S,2R)-2-aminocyclopentane-1-carboxylate (2c). Prepared using (1S,2R)-2-((tert-butoxycarbonyl)amino)cyclopentane-1-carboxylic acid (102 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.81 (t, J = 2.0 Hz, 1H), 8.70 (d, J = 2.0 Hz, 2H), 5.42 (dd, J = 30.5, 13 Hz, 2H), 3.49 (br, 1H), 3.09 (m, 1H), 1.98 (dd, J = 12.5, 7.0 Hz, 1H), 1.82 (m, 1H), 1.75 (dd, J = 24.5, 17 Hz, 2H), 1.64 (d, J = 7 Hz, 1H), 1.43 (t, J = 5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.0, 148.5 (2C), 140.8, 128.8 (2C), 118.6, 64.6, 49.0, 42.6, 27.7, 25.0, 22.6; MS (ESI): Mass calcd for $C_{13}H_{15}N_3O_6$ $[M+H]^+$ 310.09, found 310.05.



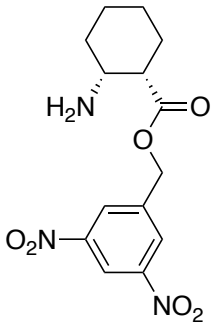
3,5-dinitrobenzyl (1S,2S)-2-aminocyclopentane-1-carboxylate (2d). Prepared using (1S,2S)-2-((tert-butoxycarbonyl)amino)cyclopentane-1-carboxylic acid (102 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, J = 2.0 Hz, 1H), 8.71 (d, J = 2.0 Hz, 2H), 5.40 (dd, J = 22.5, 13.5 Hz, 2H), 3.73 (dd, J = 13, 7 Hz, 2H), 2.56 (dd, J = 16, 7.5 Hz, 1H), 2.13 (m, 1H), 2.06 (m, 1H), 1.80 (dd, J = 14, 7 Hz, 2H), 1.73 (dd, J = 14, 7 Hz, 2H) . ^{13}C NMR (125 MHz, DMSO-d_6) ppm 173.1, 148.5 (2C), 140.8, 128.7 (2C), 118.6, 64.6, 53.8, 48.2, 31.2, 29.6, 23.6; MS (ESI): Mass calcd for $C_{13}H_{15}N_3O_6$ $[M+H]^+$ 310.09, found 310.09.



3,5-dinitrobenzyl (1R,2R)-2-aminocyclohexane-1-carboxylate (3a). Prepared using (1R,2R)-2-((tert-butoxycarbonyl)amino)cyclohexane-1-carboxylic acid (107 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t,1H, J = 2.3 Hz), 8.72 (d, 2H, J = 2.1 Hz), 5.41 (s, 2H), 3.25 (dt, 1H, J = 10.1 Hz, 3.9 Hz), 2.62 (dt, 1H, J = 11.5 Hz, 3.8 Hz), 2.02 (d, 2H, J = 9.3 Hz), 1.73-1.66 (m, 2H), 1.48-1.19 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.6, 148.5 (2C), 140.7, 128.8 (2C), 118.7, 64.8, 50.3, 46.5, 29.8, 28.5, 24.1, 23.5; MS (ESI): Mass calcd for $C_{14}H_{17}N_3O_6$ $[M+H]^+$ 324.11, found 324.07.

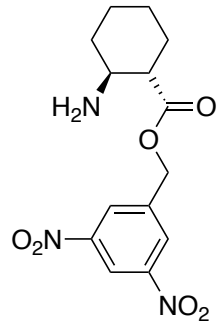


3,5-dinitrobenzyl (1R,2S)-2-aminocyclohexane-1-carboxylate (3b). Prepared using (1R,2S)-2-((tert-butoxycarbonyl)amino)cyclohexane-1-carboxylic acid (107 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, J = 2.0 Hz, 1H), 8.72 (d, J = 2.0 Hz, 2H), 5.42 (dd, J = 31.5, 13 Hz, 2H), 3.49 (m, 1H), 3.08 (m, 1H), 1.97 (m, 1H), 1.72 (m, 4H), 1.42 (m, 5H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.0, 148.5 (2C), 140.8, 128.8 (2C), 118.6, 64.6, 49.0, 42.6, 28.8, 27.7, 22.6, 21.7; MS (ESI): Mass calcd for $C_{14}H_{17}N_3O_6$ $[M+H]^+$ 324.11, found 324.05.

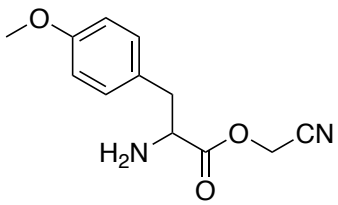


3,5-dinitrobenzyl (1S,2R)-2-aminocyclohexane-1-carboxylate (3c). Prepared using (1S,2R)-2-((tert-butoxycarbonyl)amino)cyclohexane-1-carboxylic acid (107 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). 0

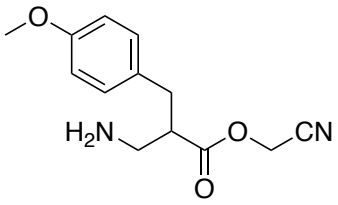
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, J = 2.0 Hz, 1H), 8.72 (d, J = 2.0 Hz, 2H), 5.42 (dd, J = 31.5, 13 Hz, 2H), 3.08 (m, 1H), 1.97 (m, 2H),1.72 (m, 5H), 1.35 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.0, 148.5 (2C), 140.8, 128.8 (2C), 118.6, 64.6, 49.0, 42.6, 27.7, 25.0, 22.6, 21.7; MS (ESI): Mass calcd for $C_{14}H_{17}N_3O_6$ [M+H]$^+$ 324.11, found 324.11



3,5-dinitrobenzyl (1S,2S)-2-aminocyclohexane-1-carboxylate (3d). Prepared using (1S,2S)-2-((tert-butoxycarbonyl)amino)cyclohexane-1-carboxylic acid (107 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), 3,5-dinitrobenzyl chloride (86 mg, 0.40 mmol) and dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, J = 2.10 Hz, 1H), 8.72 (d, J = 2.0 Hz, 2H), 5.08 (s, 2H), 3.18 (m, 1H), 2.57 (m, 1H), 2.01 (dd, J = 10.5, 7 Hz, 2H), 1.69 (m, 3H), 1.43 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.6, 148.5 (2C), 140.7, 128.8 (2C), 118.6, 64.8, 50.3, 46.5, 29.8, 28.5, 24.1, 23.5; MS (ESI): Mass calcd for $C_{14}H_{17}N_3O_6$ [M+H]$^+$ 324.11, found 324.03.



cyanomethyl 2-amino-3-(4-methoxyphenyl)propanoate (4). Prepared using 2-((tert-butoxycarbonyl)amino)-3-(4-methoxyphenyl)propanoic acid (98 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), chloroacetonitrile (26 µL, 0.40 mmol) in dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.16 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 5.11 (d, J = 2.0 Hz, 2H), 4.42 (t, J = 6.5 Hz, 1H), 3.74 (s, 3H), 3.16-3.02 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 168.8, 159.1, 131.0 (2C), 126.2, 115.6, 114.6 (2C), 55.5, 53.6, 50.6, 35.4. MS (ESI): Mass calcd for $C_{12}H_{14}N_2O_3$ [M+H]$^+$ 235.10, found 235.13.



cyanomethyl 3-amino-2-(4-methoxybenzyl)propanoate (5). Prepared using 3-((tert-butoxycarbonyl)amino)-2-(4-methoxybenzyl)propanoic acid (102 mg, 0.33 mmol), triethylamine (70 µL, 0.50 mmol), chloroacetonitrile (26 µL, 0.40 mmol) in dichloromethane (0.5 mL). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.24 (d, J = 9.0 Hz, 2H), 7.09 (d, J = 9.0 Hz, 2H), 4.95 (dd, J = 15.0, 15.0 Hz, 2H), 3.99 (s, 3H), 2.80 (m,3.47-2.94). ^{13}C NMR (125 MHz, DMSO-d_6) ppm 172.0, 159.1, 129.8 (2C), 127.1, 114.5, 113.8 (2C), 55.3, 49.2, 43.9, 40.0, 34.9; MS (ESI): Mass calcd for $C_{13}H_{16}N_2O_3$ [M+Na]$^+$ 267.11, found 267.01.

Preparation of DNA templates for RNAs

The DNA templates for flexizyme and tRNAs preparation were synthesized by using the following primers as previously described[2].

- Sequence of the final DNA templates used for *in vitro* transcription by the T7 RNA polymerase

fMet (CAU)	GTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCAACCA
Pro1E2(GGU)	GTAATACGACTCACTATAGGGTGATTGGCGCAGCCTGGTAGCGCACTTCGTTGGTAACGAAGGGGTCAGGGGTTCGAATCCCCTATCACCCGCCA

*Note that the underlined sequences are the T7 promoter sequence.

Preparation of Fx and tRNAs

Flexizymes and tRNAs were prepared using the HiScribeTM T7 High yield RNA synthesis kit (NEB, E2040S) and purified by the previously reported methods[2].

Preparation of EF-P

Expression of active EF-P[3] with beta lysilation at Lys34 requires expression of three accessory genes, YjeA (EPM-A), YjeK (EPM-B), and YfcM (EPM-C). Cds were adopted from Reference Seq NC_000913, *E. coli.* (K-12, MG1655) and ordered as Gene Blocks (IDT) for cloning into two lac expression cloning vectors, pRSFDuet-1 and pETDuet-1 with 6X His Tag at each cloning site. pRSFDuet-1 contained two genes, EF-P and EPM-A, and pETDuet-1 carried EPM-B and EPM-C. Plasmids were co-transformed into BL21 *E. coli* cells (NEB) and plated on double antibiotic (kanamycin and ampicillin) plates. Colonies were picked for overnight growth at 37°C with 250 rpm shaking in Superior Broth (AthenaES) with double antibiotic. On day 2, one liter of Superior Broth was seeded with 10mL of cells from the overnight growth, incubated at 37°C with 250 rpm shaking and induced at an OD of 0.6 with 1 mM IPTG (Promega). Cells were harvested after 4 hours and centrifuged at 4,000 g for 20 minutes in a precooled 4°C centrifuge (Beckman-Coulter Avanti J-26 XPI). Pellets were resuspended and washed in chilled Buffer I, then centrifuged again. Cell pellets were frozen at -80°C overnight. On day3, the pellets were broken up gently and resuspended in 50 mL of chilled Buffer II and transferred to 50mL Oak Ridge Tubes (Thermo-Fisher) for sonication. Cells were sonicated on ice with a 3/4 inch probe on a Sonic Dismembrator Model 500 (Fisher Scientific) for 4 minutes at 40% amplitude with 1s on/off. Sonication was repeated once, and lysate was centrifuged at 30,000 g for 30 minutes. Lysate was transferred to a 50 mL conical tube containing 500 μL of HisPur NTA Nickel Resin (Thermo Scientific) equilibrated with Buffer II and rocked gently for 30 min at 4°C. The lysate/resin mixture was pipetted into a disposable fretted 10mL polypropylene column (Thermo Scientific) and allowed to clear the column by gravity flow. Resin was

washed immediately with 75 mL of Buffer III. After washing, protein was eluted with three successive elutions of 1.5 mL of chilled Buffer IV. Elutions were transferred to a 10,000 MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Scientific) and dialyzed at 4°C in two liters of chilled Buffer V (20 mM HEPES-KOH, pH 7.0, 40 mM KCl, 1 mM $MgCl_2$, 0.1mM EDTA and 10% glycerol). After two hours, the dialysis cassette with the protein was transferred to two liters of fresh Buffer V and dialyzed overnight at 4°C. Concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific). Lysilation at Lys 34 was confirmed with 193 nm UVPD-MS for PTM localization analysis.

Buffer I: 50 mM Tris-HCl (pH 7.6), 60 mM KCl, 7mM $MgCl_2$
Buffer II: Buffer I with 7 mM β-mercaptoethanol (Sigma), 0.1mM PMSF (Sigma), and 10% glycerol (Fisher)
Buffer III: 50 mM Tris-HCl (pH 7.6), 5 mM β-mercaptoethanol, 1M NH_4Cl, 10 mM imidazole and 10% glycerol
Buffer IV: Buffer III with imidazole concentration increased to 150 mM

In vitro peptide synthesis

Below we describe the preparation of aminoacylated tRNAs and *in vitro* peptide synthesis with incorporation of non-canonical amino acids at both the N- and C- terminus of a peptide.

1) Fx-mediated acylation and purification of tRNA were performed as previously described[2].

2) N-terminus incorporation. As a reporter peptide, a T7 promoter-controlled DNA template (pJL1_StrepII) was designed to encode a streptavidin (Strep) tag and additional Ser and Thr codons (**X**WSHPQFEKST (strep-tag), where **X** indicates the position of the cβAA substrate). The translation initiation codon AUG was used for N-terminal incorporation of the cβAA substrates, **X**. Peptide synthesis was performed using only the 9 amino acids that decode the initiation codon AUG and the purification tag in the absence of the other 11 amino acids to prevent corresponding endogenous tRNAs from being aminoacylated and used in translation. The PURExpress® Δ (aa, tRNA) kit (NEB, E6840S) was used for polypeptide synthesis reaction and the reaction mixtures were incubated at 37 °C for 3 h. The synthesized peptides were then purified using Strep-Tactin®-coated magnetic beads (IBA), denatured with SDS, and characterized by MALDI-TOF mass spectroscopy.

3) C-terminus incorporation. The same plasmid (pJL1-StrepII) encoding the same amino acids (MWSHPQFEKS**X**, where **X** indicates the position of the cβAA substrate) was used for C-terminal incorporation and the cβAA substrate was incorporated into the Thr codon (ACC) using the same kit. 10

μM (final concentration) of the EF-P was added to the reaction mixture for the C-terminal cβAA incorporation.

Purification of peptide products

The polypeptides containing a cβAA were purified using an affinity tag purification technique as previously described[2].

Supplementary Figures

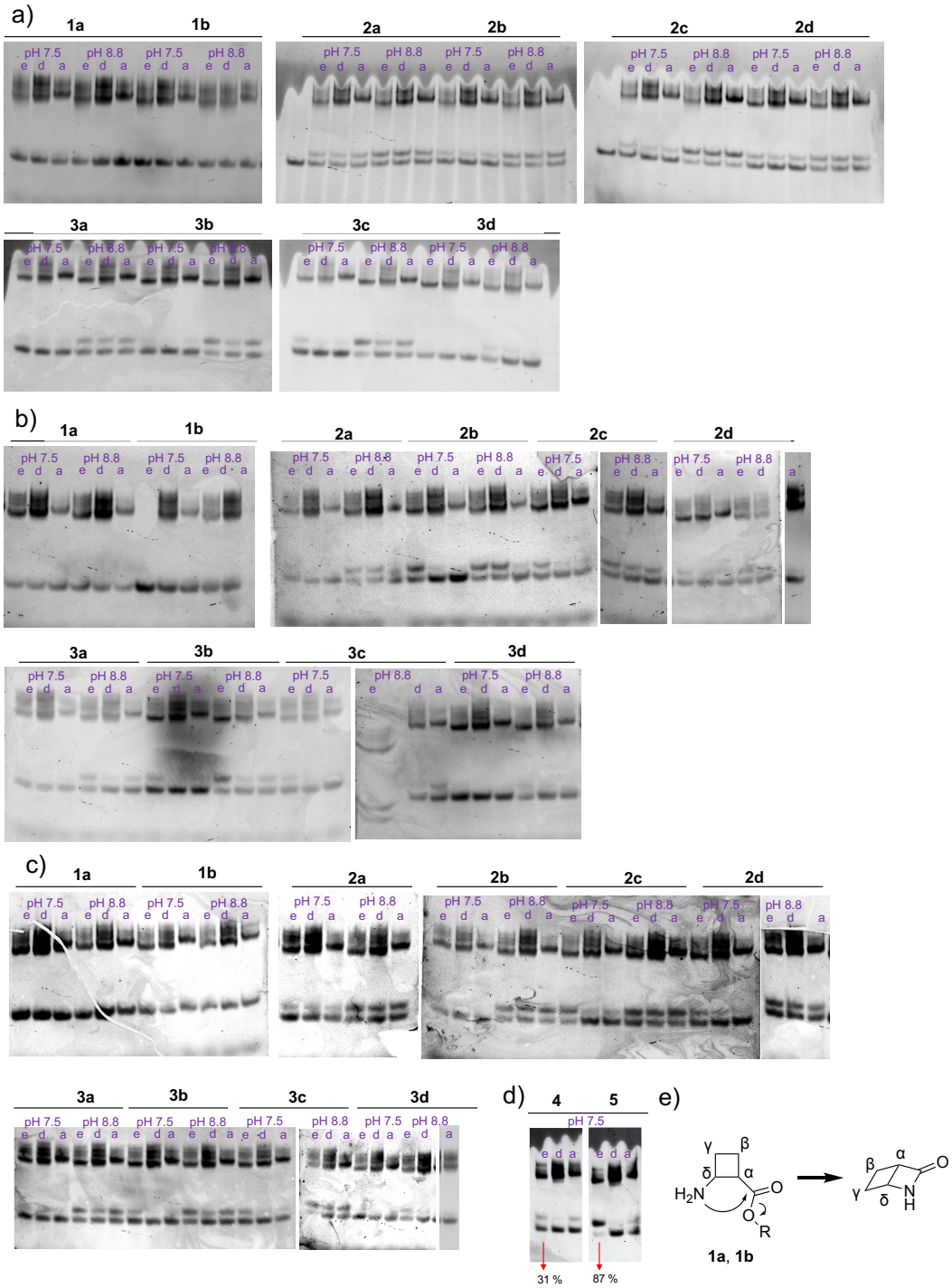


Figure S1. Acylation of microhelix with substrates 1-5. The Fx-catalyzed acylation reaction using the 12 substrates were monitored at two different pH (7.5 or 8.8) over 24 h with three different flexizymes (eFx, dFx, and aFx). The yield of each reaction was determined by quantifying the relative band intensity of unacylated and acylated microhelix on the gel using ImageJ software (a-d). Proposed mechanism of cyclic lactam formation for substrate **1a** and **1b**[4-6] (e).

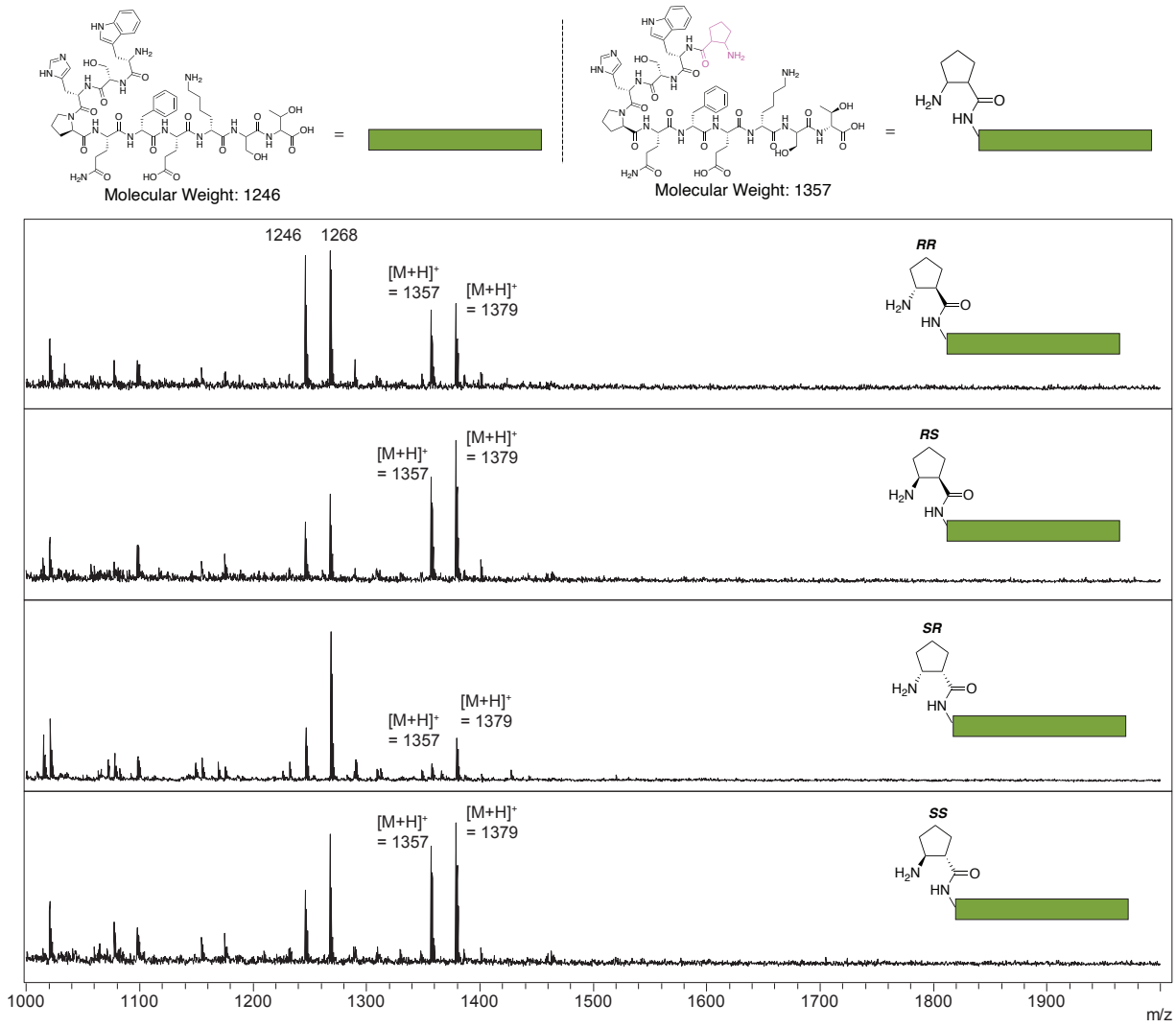


Figure S2. Characterization of the N-terminus functionalized peptide with 5-cβAAs (2a-2d).
The sequence of green peptide is WSHPQFEKST, which corresponds to the theoretical mass of a peptide not bearing the substrate at the N-terminus, $[M+H]^+$ = 1246; $[M+Na]^+$ = 1268. All the 5-cβAAs (**2a**-**2d**) are found to be incorporated into the N-terminus by the natural translation machinery. $[M+H]^+$ = 1357; $[M+Na]^+$ = 1279. The relative yields of a target polypeptide containing **2a**, **2b**, **2c**, and **2d** at the N-terminus are approximately 36, 64, 18, and 57 %, respectively. The yield was calculated based on the relative peak area of the target polypeptides shown in the spectrum.

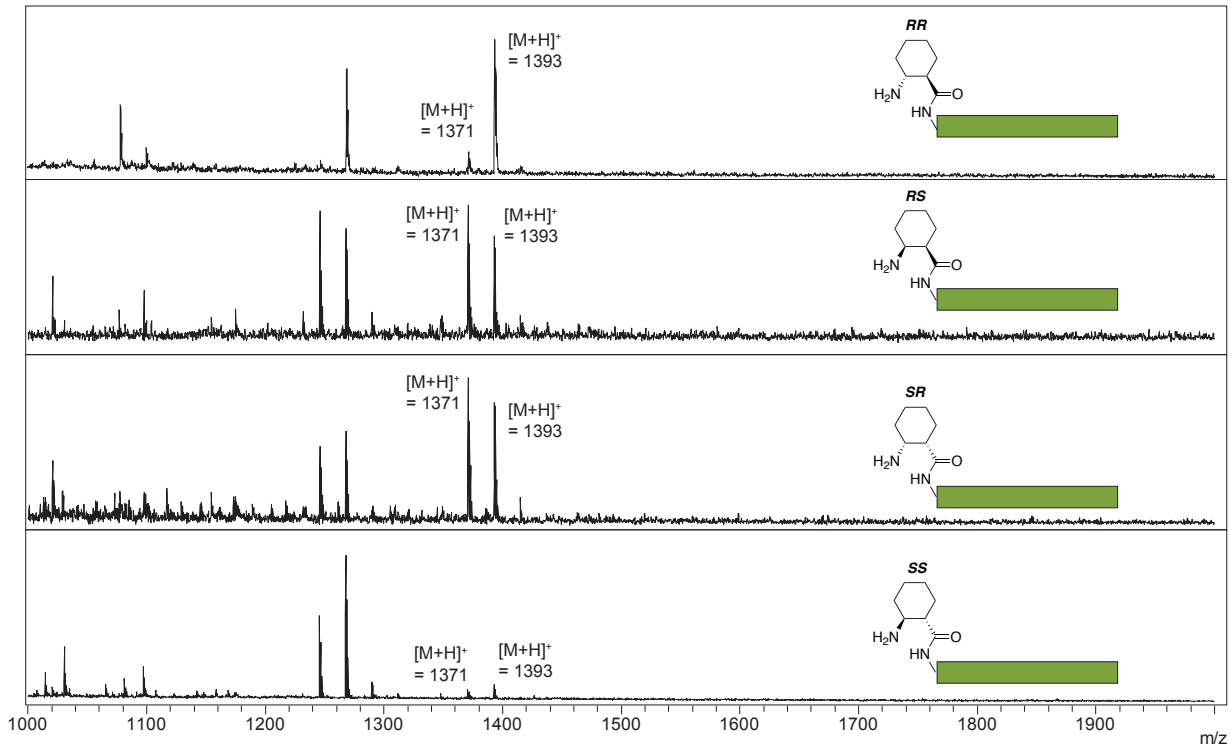


Figure S3. Characterization of the N-terminus functionalized peptide with 6-cβAAs (3a-3d).
All the 6-cβAAs (**3a**-**3d**) are found to be incorporated into the N-terminus by the natural translation machinery. $[M+H]^+$ = 1371; $[M+Na]^+$ = 1393. The relative yields of a target polypeptide containing **3a**, **3b**, **3c**, and **3d** at the N-terminus are approximately 59, 48, 63, and 7 %, respectively.

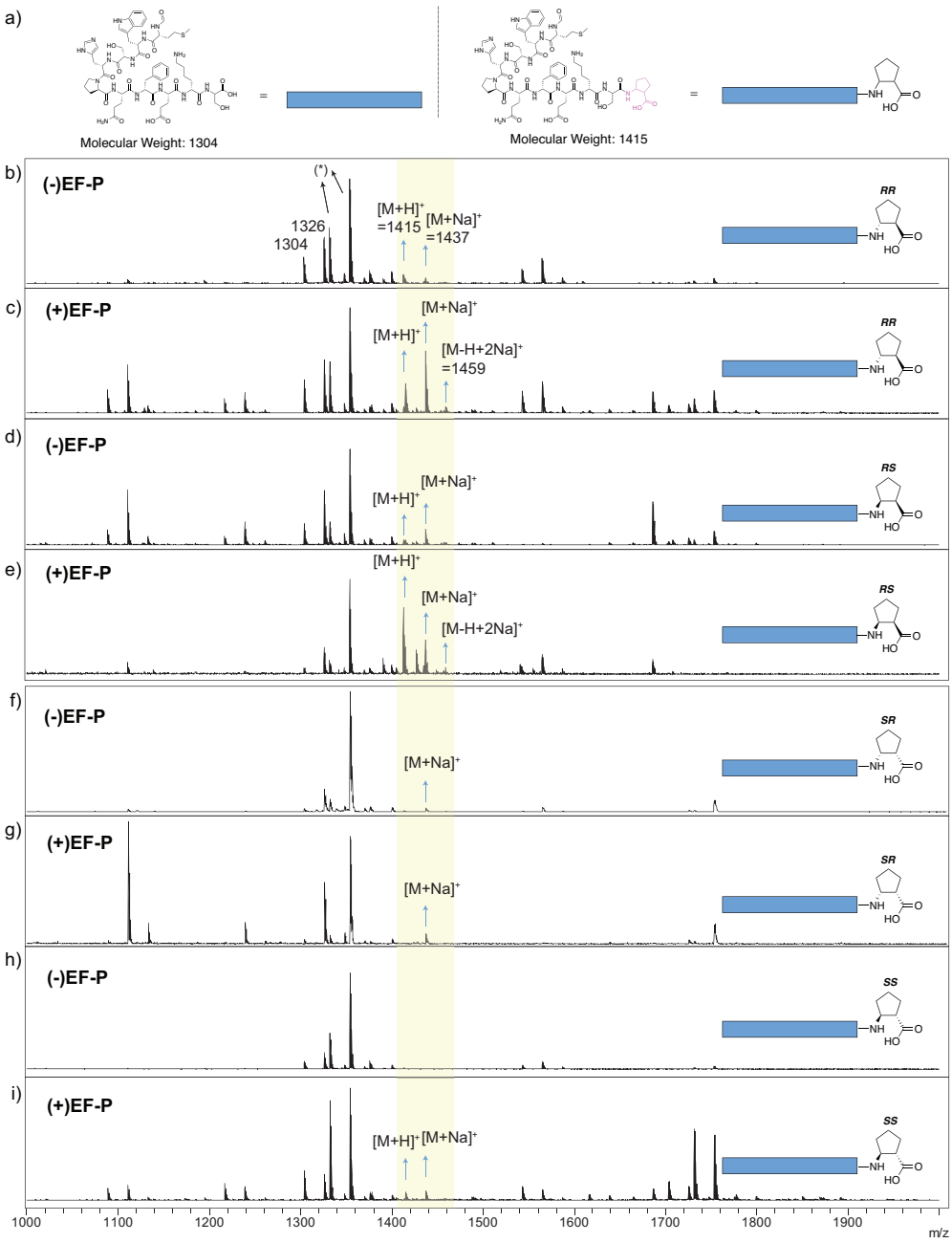


Figure S4. Addition of EF-P enhances C-terminus incorporation of 5-cβAAs (2a-2d) into a target polypeptide. Addition of EF-P (c, e, g, and i) under the same reaction condition in PURExpress™ yielded a peak with enhanced intensity that is corresponding to the theoretical mass of a peptide containing the 5-cβAA substrate into the C-terminus. The theoretical mass of peptide is $[M+H]^+$ = 1415; $[M+Na]^+$ = 1437; $[M-H+2Na]^+$ = 1459. The sequence of blue peptide is fMWSHPQFEKS, which corresponds to the theoretical mass of a peptide not bearing the substrate at the C-terminus, $[M+H]^+$ = 1304; $[M+Na]^+$ = 1326. The truncated peptide (mass: 1304 Da) is produced, presumably because the ribosomal synthesis comes to completion earlier than the incorporation of rigid cβAA at the C-terminus. The marked peaks by an asterisk ($[M+H]^+$ = 1334; $[M+Na]^+$ = 1356) were unidentified. The highlighted (yellow) area was used to produce **Fig. 4a-b.**

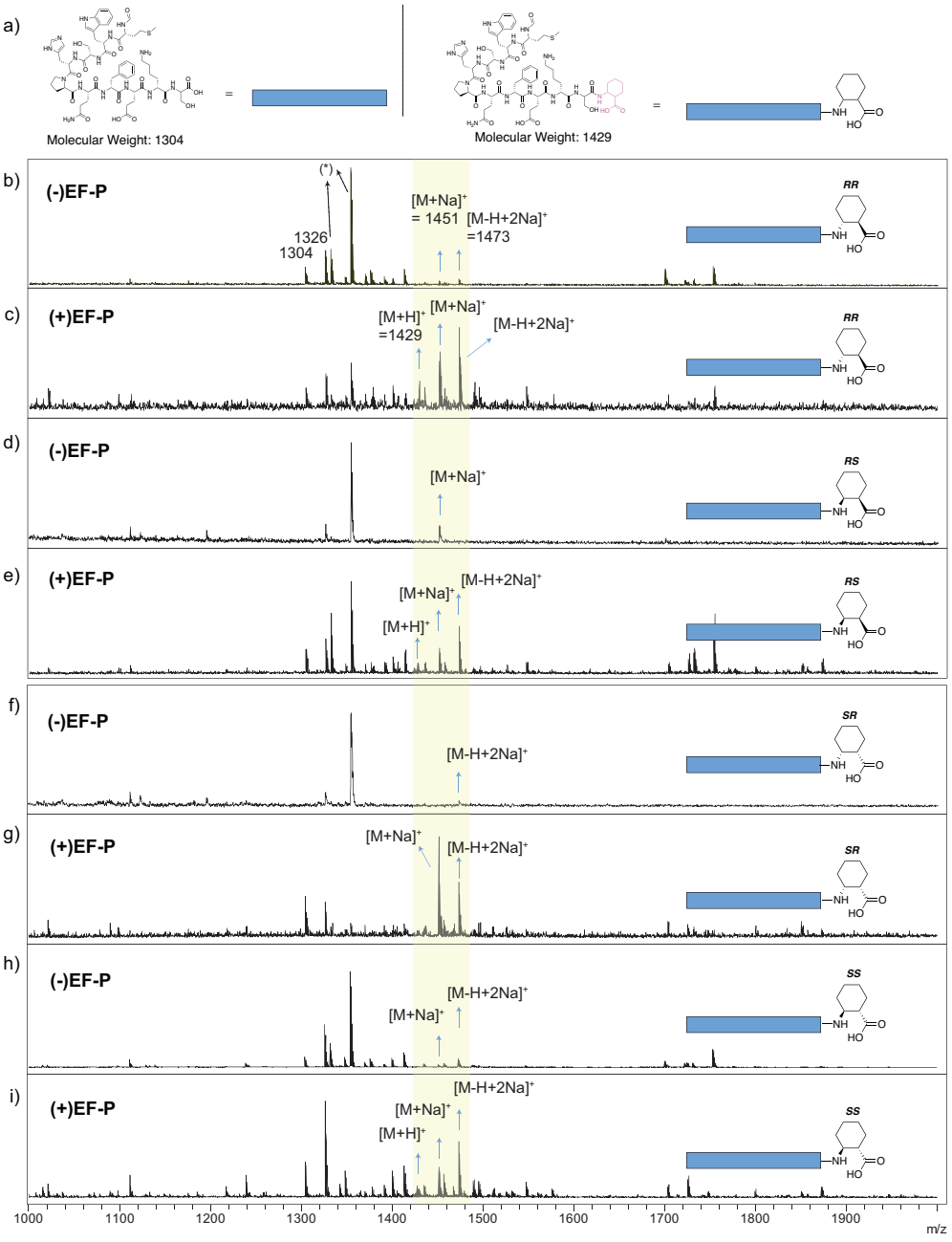


Figure S5. Addition of EF-P increases C-terminal incorporation of 6-cβAAs (3a-3d) into a target polypeptide. Addition of EF-P (c, e, g, and i) under the same reaction condition in PURExpress™ yielded an enhanced peak of that is corresponding to the theoretical mass of a peptide containing a 6-cβAA substrate into the C-terminus. The theoretical mass of peptide is $[M+H]^+$ = 1429; $[M+Na]^+$ = 1451; $[M-H+2Na]^+$ = 1473. The sequence of blue peptide is fMWSHPQFEKST, which corresponds to the theoretical mass of a peptide not bearing the substrate at the C-terminus, $[M+H]^+$ = 1304; $[M+Na]^+$ = 1326. The highlighted (yellow) area was used to produce **Fig. 4c-d.**

a)

	2a			2b			2c			2d		
	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+
(-)EF-P	77	47	-	42	166	-	-	34	-	14	-	-
(+)EF-P	274	585	52	642	326	63	-	89	-	69	84	-

	3a			3b			3c			3d		
	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+	[M+H]+	[M+Na]+	[M-H+2Na]+
(-)EF-P	-	63	101	-	154	-	-	-	29	-	-	69
(+)EF-P	1000	1444	1778	-	288	519	-	4600	2800	139	316	734

b)

$$CIE(\%) = \frac{\Sigma \text{ of PA (fMWSHPQFEK}\mathbf{c\beta AA}\text{)}}{\{\Sigma \text{ of PA (fMWSHPQFEK)} + \Sigma \text{ of PA (fMWSHPQFEK}\mathbf{c\beta AA}\text{)}\}} \times 100$$

	2a	2b	2c	2d	3a	3b	3c	3d
(-)EF-P	16.0	19.9	12.0	5.7	2.9	53.1	23.9	17.3
(+)EF-P	53.1	70.3	12.6	23.8	73.5	57.5	67.1	46.5

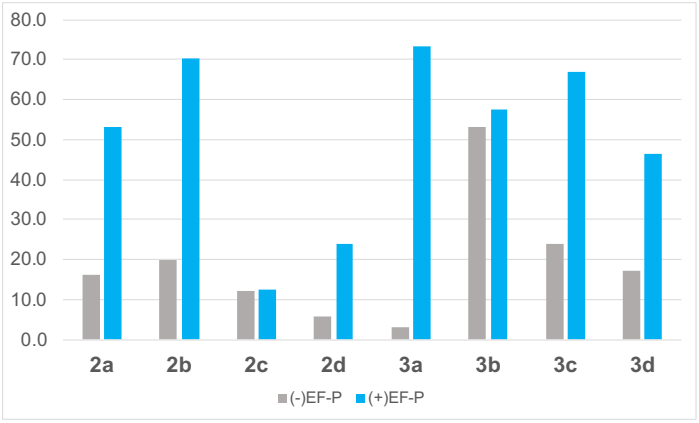


Figure S6. Analysis of the C-terminal incorporation of cβAA.
a) The addition of EF-P under the same reaction condition in PURExpress™ yielded an enhanced signal for all the peaks corresponding to the theoretical mass of peptide containing a cβAA (**2a**-**2d** and **3a**-**3d**) at the C-terminus. This suggests the amount of the target peptides is increased in the sample. The signal-to-noise ratio (S/N) was normalized using S/N of the peak at 1353 present in all the spectrum as an internal reference, then multiplied by an arbitrary number (1,000) to compare the peak signals in **Fig. 4** quantitively. b) The C-terminal incorporation efficiency (CIE, %) was determined based on the relative peak area (PA) of a target polypeptide over a total amount of the truncated and target polypeptides. The incorporation efficiency of cβAA is enhanced by approximately 0.6 to 70.6 % depending on the monomer after the addition of EF-P. The signal-to-noise (S/N) ratio and the peak area was processed with Compass DataAnalysis 4.2 software (Bruker).

Plasmid map

Below is the sequence for the gene encoding target polypeptide with a cβAA either at the N- or C-terminus.

GGATCCTGCAGTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGAT
CAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCAC
CACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACC
GGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACC
TACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGG
GTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGT
CGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAACGAATTCAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGAC
CACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA[***CATATG***TGGTCTCATCCGCAGTTCGAAAAATCCACCTAG
TAA***GTCGAC***]CGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCT
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TTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGG
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GCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCTTCGAGCAAGACGTT
TCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATCTTGTGCA
ATGTAACATCAGAGATTTTGAGACACAACGT

***CATATG*: NdeI**

***GTCGAC*: SalI**

TGGTCTCATCCGCAGTTCGAAAAA: strep tag

TAGTAA: stop

>pJL1_StrepII

[*CATATG*TGGTCTCATCCGCAGTTCGAAAAATCCACCTAGTAA*GTCGAC*]

fMetTrpSerHisProGlnPheGluLysSerThr

References

1. Pangborn, A.B., Giardello, M.A., Grubbs, R.H., Rosen, R.K. & Timmers, F.J. Safe and convenient procedure for solvent purification. *Organometallics* **15**, 1518-1520 (1996).
2. Lee, J. et al. Expanding the limits of the second genetic code with ribozymes. *Nat Commun* **10**, 5097 (2019).
3. Peil, L. et al. Lys34 of translation elongation factor EF-P is hydroxylated by YfcM. *Nat Chem Biol* **8**, 695-697 (2012).
4. Ohshiro, Y. et al. Ribosomal synthesis of backbone-macrocyclic peptides containing gamma-amino acids. *Chembiochem* **12**, 1183-1187 (2011).
5. Terasaka, N., Iwane, Y., Geiermann, A.S., Goto, Y. & Suga, H. Recent developments of engineered translational machineries for the incorporation of non-canonical amino acids into polypeptides. *Int J Mol Sci* **16**, 6513-6531 (2015).
6. Lee, J., Schwarz, K.J., Kim, D.S., Moore, J.S. & Jewett, M.C. Ribosome-mediated polymerization of long-carbon chain and cyclic amino acids into peptides in vitro. *submitted* (2020).