Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Spontaneous co-translational peptide macrocyclization using *p*-cyanoacetylenephenylalanine

Osorio Estheban[a,b], Brennan Chaloux[a,b], Hartman Matthew[*a,b]

[a] Massey Cancer Center, Virginia Commonwealth University, 401 College St, Richmond,
23219, VA, United States
[b] Department of Chemistry, Virginia Commonwealth University, 1001 W Main Street, Richmond, 23284, VA, United State

* Corresponding author

Email: osoriofranche@vcu.edu (Estheban Osorio), chaloubt@vcu.edu (Brennan Chaloux), *mchartman@vcu.edu (Matthew C. T. Hartman)

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Materials

Salts and solvents: Salts and NTPs (ATP, GTP,CTPand UTP) were purchased from Fisher scientific, Sigma-Aldrich and Thermo-Fisher. Mix of buffered Phenol/$CHCl_3$/Isoamylalcohol was purchased from Acros Organics. Antibiotics were purchased in Gentrox and Fisher scientific. Natural amino acids were purchased from Fluka and dissolved in water to 10mM, pH was adjusted to 7.2-7.6 with KOH and sterile filtered (0.22µm) for subsequent storage at -20°C.

Instruments: DNA, RNA, Optical density, and protein concentrations were quantified through UV-Vis Eppendorf BioPhotometer Model #6131. pH was measured with Mettler Toledo SevenEasy S20 pH Meter. Mass spectrometry was carried through Voyager DE-Pro MALDI-TOF, samples were prepared with α-Cyano-4-hydroxycinnamic acid purchased through Millipore-Sigma (C2020-10G). High-resolution mass spectrometry was done through AccuTOFMS in positive mode using DART helium stream as ion source; samples were dipped in a capillary tube prior to introduction into the helium stream. DNA was made with Biorad Dual 48 Well DNA Engine Thermal Cycler. Radioactive samples were measured with a Beckman Coulter LS6500 Liquid Scintillation Counter. Benchtop centrifuges are Sorvall legend micro 17 and for larger volumes Sorvall Superspeed RC2-B was used.

Experimental Methods

PURE System purification: All amino-acyl synthetases and protein factors were expressed as follows; genes were cloned into pET-28a/pET-16b/pET-21/pET-20a/pET-24/pQe30/pET3a plasmids with C-Terminal hexahistidine tag, the resulting plasmids were transformed into Mach1 and BL21 cells. The enzymes were expressed in LB media with 30µg/mL of kanamycin or 1000 µg/mL of ampicillin respectively. The cells were induced at OD=0.6 with 0.1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) (BioBasic Cat# IB0168). Cells were pelleted down at 3000 x g for 20min at 4°C and lysed using detergent-based B-PER in Phosphate buffer (ThermoFisher Scientific Cat# 78266) in the presence of protease inhibitor cocktail (ThermoFisher Scientific Cat# P8849-5ML). After 15 min of shaking at room temperature (rt), debris was pelleted by centrifugation at 25000 x g for 15min at 4°C, all further purification steps were done at this temperature. Supernatant was transferred to a 20mL chromatography column (Bio-Rad Cat# 7321010) with 1mL of Ni-NTA agarose beads (MCLab Cat# Ni-NTA-300) and left 1 hour for binding. Column was drained and washed with wash buffer 2x10mL (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, 5mM β-Mercaptoethanol (BME)) and eluted with 6x1mL of elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, 5mM BME). The enzyme containing fractions were dialyzed overnight with 50mM HEPES-KOH, pH 7.6, 100mM KCl, 10mM $MgCl_2$, 7mM BME and 30% glycerol, quantified and stored at -80°C except for MetRS which was stored at -20°C with glycerol concentration of 50%. Ribosomes were produced as described before[1].

Charging of pCAF to $tRNA^{Phe}$: The charging assay was carried out as described in previous publications[2]. Briefly, buffered pCAF (100-500µM pH=7.4) was mixed with HEPES/KOH (30mM pH=7.4), *E. coli* total tRNA (10mg/mL) (Roche Cat#: 10109550001), $MgCl_2$ (15mM), KCl (25mM), ATP (6mM), inorganic pyrophosphatase (0.001mg/mL) (Sigma-Aldrich Cat#: I5907-1MG), PheRS (A294G) (0.3-0.51µM) and bovine serum albumin (BSA, 0.24 mg/mL) previously dialyzed into deionized water (dH_2O) in a final volume of 50µL. Reactions were also incubated with or without β-Mercaptoethanol (20 or 200mM). The reaction was incubated for 30-60 min at

37°C before quenching by adding 0.1 volumes of aq. NaOAc (3M pH=5.2). The RNA-containing aqueous phase was extracted by mixing it with a mixture of phenol/$CHCl_3$/isoamyl alcohol (25/24/1) (unbuffered) and subsequently vortexed and centrifuged to separate layers. Then aqueous layer was removed and transferred to a clean vial and mixed with an equal volume of $CHCl_3$ and vortexed and centrifuged again. The aqueous layer was removed into a clean vial and mixed with 3 volumes of ice-cold ethanol. The vial was incubated at -20°C for 20 min and centrifuged for 20 min at 4°C at 17000 x *g*. The pellet was washed with 500µL of 70% Ethanol and then 500µL of 100% ethanol to remove salts. Finally, the pellet was left for air dry and resuspended in NaOAc (12.5µL, 100mM pH=5.0). Reductive amination was carried out by mixing 6.25µL of the previous tRNA-aa preparation with 3.75µL of dH_2O, (4-formylphenoxypropyl)triphenylphosphonium bromide in MeOH (12.5 µL, 63 mM) and fresh $NaBH_3CN$ dissolved in 50mM NaOAc pH=5.0 (2.5 µL, 200mM). The reaction was incubated at 37°C on tumbler for 2 hours before quenching with 0.1 volume of NH_4OAc (4.4M pH=5.0). The reaction product was recovered through ethanol precipitation and the resulting pellet was resuspended in of NH_4OAc (2.25µL, 200mM pH=5.0). 0.25µL of Nuclease P1 (1U/µL in 200 mM NH_4OAc pH=5.0) (Wako Cat#: 145-08221) was added and incubated at rt for 20 min. After incubation, the reaction mixture was quenched on ice and 1µL was mixed with 9µL of MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) 10mg/mL in MeCN:2% TFA (1:1) and spotted onto the MALDI plate for further analysis.

In vitro transcription of mRNAs: UniFWD and UniREV primers (1µM each) are mixed with the custom oligo templates (0.01µM), Q5 reaction buffer (1X), dNTPs (0.2mM), Q5 High-Fidelity DNA Polymerase (20U/mL) and dH_2O to complete 500µL reaction and allowed to PCR amplify according to manufacturers recommended protocol for 15 cycles. Then DNA was solvent extracted (unbuffered phenol) and ethanol precipitated as explained above. The DNA pellet was dissolved in 100µL of water and mixed with transcription mix composed by: Tris/Triton (40mM pH=7.8), Spermidine (2.5mM), $MgCl_2$ (25mM), Dithiothreitol (10mM), UTP/GTP/CTP/ATP (5mM each), extra GTP (4mM), RiboSafe RNase Inhibitor (0.2U/µL Bioline Cat#: C755H60), T7 Polymerase (0.2µM) and inorganic pyrophosphatase (1µ/mL) in a final volume of 500 µL. The transcription was incubated overnight and further purified through urea denaturing gel electrophoresis followed by extraction methods, either electroelution or salt crush/soak. Then mRNAs were quantified by UV-Vis absorbance and diluted to 30 µM.

In the case of Pep3 and Pep9, DNA was amplified from a template that was cloned in pET12b with the desired sequences flanked with the standard T7 promotor and T7 terminator regions. The PCR protocol followed is the same but T7FWD and T7REV primers were used instead.

The mRNA used for Pep10 is described in David Hacker, oligos and strategy for transcription can be seen in the following reference named as 9.4b[3].

Primers and sequences are included in **Supporting Tables 1 and 2**.

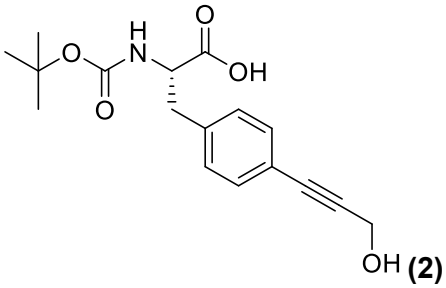
***In vitro* translation experiments:** Experiments were carried in absence of PheRS and phenylalanine, PheRS(A294G) was used instead. Translation started with the mix of the following components: dH_2O, TS-Solution (HEPES-KOH pH=7.6 (50mM), AcOK (100mM), $MgOAc_2$ (6mM), Spermidine (10mM), Dithiothreitol (1mM) or TCEP (125mM), Creatine Phosphate (20mM), 10-Formyltetrahydrofolate (100µM), ATP/GTP (1.5mM each, potassium exchanged), total *E. coli* tRNA (100mg/Ml, deacylated and dialyzed overnight against 50mM Tris/HCl pH=9, precipitated and resuspended in dH_2O), Factor Mix (EF-G (0.52µM), IF-1

(2.7μM), IF-2 (0.4μM), IF-3 (1.5μM), MTF (0.6μM), RF-1 (0.3μM), RF-3 (0.17μM), RRF (0.5μM), EF-Tu (10μM), EF-Ts (4.1μM), Ribosomes (1.2μM), amino acids required for the template except methionine (12.5-100μM each, pCAF was used between 100-500 μM), aminoacyl-tRNA synthetases (0.03-1.23μM), FRS A294G was used at 0.51μM, inorganic pyrophosphatase (1μg/mL), Creatine Kinase (4μg/mL), Myokinase from rabbit (3μg/mL) (Sigma-Aldrich Cat#: M3003-2.5KU), Nucleoside 5′-Diphosphate Kinase (1.1μg/mL) (Sigma-Aldrich Cat#: N2635-100UN). To the resulting mix, methionine (10μM) and ^{35}S-methionine (optional) (0.1μM) was added simultaneously followed by the desired mRNA template (1μM). For negative controls the template was left out. The reaction mixture was incubated for 1 hour at 37°C and quenched with wash buffer (50mM Tris-HCl, 300mM NaCl). Then, the reaction was transferred to a centrifugal filter (VWR Cat#: 82031-358) and mixed with with 10μL of anti-FLAG M2 agarose beads (Sigma-Aldrich Cat#: A2220-1ML) or 40μL of Ni-NTA resin (MCLabs Cat#: NINTA-500) and left for binding for 1 h with tumbling at rt After binding, the supernatant was removed by centrifugation (1 min, rt, 7800 x g) and washed 3X500μL with wash buffer (50mM Tris-HCl, 300mM NaCl at pH=8). Elution was done by incubating the beads 15 min with 50μL of trifluoroacetic acid 1% in dH_2O, at rt and subsequently centrifuged (1 min, rt, 7800 x g). The flowthrough was used in two different ways. For samples without radioactive methionine, flowthrough was desalted with a homemade zip-tips (Empore™ Cat#: 76333-132, Rainin Cat#: 17014048), and eluted with CHCA 10mg/mL in MeCN:0.2% TFA (1:1), spotted in the MALDI-TOF plate and analyzed. For radioactive samples, 30μL of the flowthrough was mixed with 2mL of scintillating fluid and analyzed with a scintillating counter for quantification.

Protease digestion: Digestion experiments of *in-vitro* translated peptides proceeded after the elution step mentioned in the "***In vitro* translation experiments"** section. 1 volume of protease buffer (40mM Tris-HCl), 200mM NaCl, 4mM $CaCl_2$,pH=8.0) and 1 μL of protease Factor Xa (New England Biolabs Cat#: P8010S) is added to the eluted peptide. The sample was incubated at rt for 18 h and the protease was inactivated by heating at 70°C for 10 min. Then, 3 volumes of wash buffer were added and further binding, washes, elution and MALDI-TOF analysis of the digested peptide were carried out as explained above.

pCAF synthesis

Synthesis of (R)-2-((tert-butoxycarbonyl)amino)-3-(4-(3-hydroxyprop-1-yn-1-yl)phenyl)propanoic acid (2)



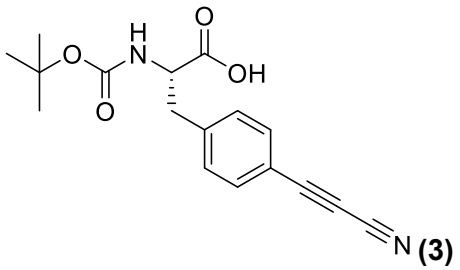
(2)

Boc-L-iodophenylalanine (493.4mg, 1.26mmol) was dissolved in of $(iPr)_2Net$ (1.76mL, 10.08mmol) and 4.1 mL of THF. Then, CuI (3.9mg, 0.02mmol,) and $(Ph_3P)_2PdCl_2$ (9.6mg, 0.05mmol) were added under Ar atmosphere for 5 minutes. Once homogenized, prop-2-yn-1-ol

(84.8mg, 1.51mmol) was added and the solution was stirred for 5 h. Then, the reaction mixture was purified through flash chromatography using hexane/AcOEt/AcOH (6.8/3.2/1) as the mobile phase. Fractions were analyzed with TLC and those who had the product were evaporated under reduced pressure giving the title compound as orange oil (0.31g, 77% yield).

^{1}H-NMR: δ= 7.32 (2H, m), 7.23 (2H, m), 7.03 (1H, d, *J=8.4* Hz), 4.28 (2H, S), 4.08 (1H, ddd, br J=4.5Hz, *J*=4.2Hz, *J*=10.5Hz), 3.02 (1H, dd, *J*=4.5Hz, *J*=13.6Hz), 2.82 (1H, dd, *J*=10.5Hz, *J*=13.6Hz), 1.31 (9H, s). **^{13}C-NMR:** δ= 173.3036, 155.40, 138.72, 130.97, 129.43, 120.35, 89.47, 83.55, 77.99, 59.72, 49.43, 36.34, 28.12. **HRMS** (AccuTOFMS-DART): m/z $[M+H]^+$ calculated for $C_{17}H_{22}NO_5^+$ = 320.1492; observed = 320.1537. $[M - Boc + H]^+$ m/z calculated for $C_{12}H_{14}NO_3^+$ = 220.0968; observed = 220.0956.

Synthesis of (R)-2-((tert-butoxycarbonyl)amino)-3-(4-(cyanoethynyl)phenyl)propanoic acid (3)

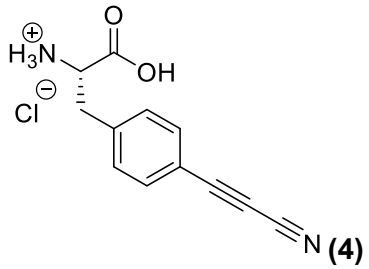


(3)

In a round bottom flask, Boc-L-(4-propargyloxy)Phenylalanine (150mg, 0.38mmol), compound **2**, was dissolved in MeCN/dH_2O (9/1, 1.35mL) which was subsequently mixed with ammonium acetate (118mg, 1.53mmol) and TEMPO (3.0mg, 0.02mmol). After 5 minutes, Diacetoxyiodobenze (271.10mg, 0.84 mmol) was added to the reaction mixture and under argon atmosphere. The reaction was stirred for 90 minutes and was quenched by the addition of Et_2O (1.5mL) and dH_2O (0.5mL). The organic phase was evaporated, and the product was purified through flash chromatography using hexanes/AcOEt/AcOH (6.8/3.2/1) giving the title compound as a yellowish solid (0.120g, 99.6% yield).

^{1}H-NMR: δ=7.73 (2H, m), 7.40 (2H, m), 7.13 (1H, d, *J=8.8*Hz), 4.13 (1H, ddd, *J=4.5*Hz, *J=8.8*Hz, *J=10.8*Hz,) , 3.10 (1H, dd, *J*=4.5Hz, *J*=13.8Hz), 2.89 (1H, dd, *J*=10.8Hz, *J*=13.4Hz), 1.30 (9H, s). **^{13}C-NMR:** δ= 173.13, 155.35, 143.60, 133.58, 130.05, 114.00, 105.45, 83.97, 78.07, 62.01, 36.57, 28.08. **HRMS** (AccuTOFMS-DART): $[M+H]^+$ m/z calculated for $C_{17}H_{19}N_2O_4^+$ = 315.1339; observed = 315.1344 $[M+H]^+$. $[M - Boc + H]^+$ m/z calculated for $C_{12}H_{14}NO_3^+$ = 215.0815; observed = 215.0815

Synthesis of (R)-2-amino-3-(4-(cyanoethynyl)phenyl)propanoic acid (*p*-cyanoacetylene-phenylalanine, pCAF, 4)



(4)

Boc-N-(4-propiolonitrile)-L-phenylalanine (60mg, 0.19mmol) was added to a round bottom flask and mixed with 10mL of HCl (4M)/AcOEt (3.28mL of conc. HCl and 8.72mL of AcOEt). The mixture was stirred for 20 min and then was evaporated leaving an oily residue. Et_2O (3x10mL) was added, resulting in the precipitation of the desired product as a white powder 47.2mg (98.7% yield).

^{1}H-NMR: δ= 7.77 (2H, m), 7.46 (2H, m), 4.18 (1H, m), 3.23 (2H, d, *J*=6.13). **^{13}C-NMR:** δ= 170.02, 14.58, 133.92, 130.44, 114.86, 105.41, 83.71, 62.25, 35.62. **HRMS** (AccuTOFMS-DART): $[M+H]^+$ m/z calculated for $C_{12}H_{11}N_2O_2^+$ = 215.0815; observed = 215.0816.

Name	Sequence (5' to 3')
UniFWD	GGCGTAATACGACTCACTATAGGGTTAACTTTACCGAAGGAGGAAAGA
Pep1 TOP	CCGAAGGAGGAAAGAATGGCGTTTGCGATGTGCAGCAGCGCG
Pep1 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCGUUUGCGAUGUGCAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
Pep2 TOP	CCGAAGGAGGAAAGAATGGCATTTGCTATGGCAAGCAGCGCG
Pep2 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCAUUUGCUAUGGCAAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
Pep5 TOP	CCGAAGGAGGAAAGAATGGCGTTTGCGATGGCGAGCTGCAGCAGCGCG
Pep5 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCGUUUGCGAUGGCGAGCUGCAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
Pep6 TOP	CCGAAGGAGGAAAGAATGGCGTTTGCGATGGCGAGCGCGAGCTGCAGCAGCGCG
Pep6 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCGUUUGCGAUGGCGAGCGCGAGCUGCAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
Pep7 TOP	CCGAAGGAGGAAAGAATGGCCTTTGCTATGGCTAGTGCAAGCGCATCCTGCAGCAGCGCG
Pep7 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCCUUUGCUAUGGCUAGUGCAAGCGCAUCCUGCAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
Pep8 TOP	CCGAAGGAGGAAAGAATGGCATTTGCAATGGCCTCTGCCTCGGCCTCAGCGTCATGCAGCAGCGCG
Pep8 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGGCAUUUGCAAUGGCCUCUGCCUCGGCCUCAGCGUCAUGCAGCAGCGCGGAUUAUAAAGACGACGAUGACAAAUAGUAG
UniREV	CTACTATTTGTCATCGTCGTCTTTATAATCCGCGCTGCT
Xa Protease Control	GGTTAACTTTACCGAAGGAGGAAAGAATGCATAGCATTGAAGGCCGCTTTGATTATAAAGATGATGATGATAAATAGTAG
Pep4 mRNA	GGUUAACUUUACCGAAGGAGGAAAGAAUGCAUAGCAUUGAAGGCCGCUUUGAUUAUAAAGAUGAUGAUGAUAAAUAGUAG
Xa Reverse Primer	CTACTATTTATCATCATCATCTTTA

Supporting Table 1. DNA oligos used for the preparation of the mRNA templates

Name	Sequence (5' to 3')
T7FWD	TAATACGACTCACTATAGGG
Pep3 DNA	TAATACGACTCACTATAGGG AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGCATTGCATTGAAGGCCGTTTTGATTATAAAGACGACGATGACAAAT AGTAGGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGC
Pep3 mRNA	GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUUUAACUUUAAGAAGGAGAUAUACCAUGCAUUGCAUUGAAGGCCGUUUUGAUUAUAAAGACGACGAUGACAA AUAGUAGGGAUCCGGCUGCUAACAAAGCCCGAAAGGAAGCUGAGUUGGCUGCUGCCACCGCUGAGCAAUAACUAGC
Pep9 DNA	TAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGCATTGCCATAAAATGTTTGATTATAA AGACGACGATGACAAATAGTAGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCG
Pep9 mRNA	GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUUUAACUUUAAGAAGGAGAUAUACCAUGCAUUGCCAUAAAAUGUUUGAUUAUAAAGACGACGAUGACAAAUA GUAGGAUCCGGCUGCUAACAAAGCCCGAAAGGAAGCUGAGUUGGCUGCUGCCACCGCUGAGCAAUAACUAGCG
T7REV	GCTAGTTATTGCTCAGCGG

Supporting Table 2. Primers used to prepare mRNAs from plasmid DNA.

Supporting Table 3. Peptides with pCAF and their masses

Name	Peptide	Expected [M+H]+	Observed [M+H]+	Expected + BME Michael [M+H]+	Observed + BME Michael [M+H]+
Pep1	fMApCAFAMCSSADYKDDDDK	1989.73	1989.80	NA	
Pep2	fMApCAFAMASSADYKDDDDK	1957.76	1958.11	2035.77	2036.11
Pep3	fMHCIEGRpCAFDYKDDDDK	2063.82	2064.07	NA	
Pep4	fMHSIEGRpCAFDYKDDDDK	NA		2125.86	2125.81
Pep5	fMApCAFAMASCSSADYKDDDDK	2147.80	2147.93	NA	
Pep6	fMApCAFAMASASCSSADYKDDDDK	2305.87	2305.93		
Pep7	fMApCAFAMASASASCSSADYKDDDDK	2463.94	2463.98		
Pep8	fMApCAFAMASASASASCSSADYKDDDDK	2622.01	2622.22		
Pep9	fMHCHKMpCAFDYKDDDDK	2004.77	2004.81		
Pep10	fMHPQNCpCAFHVpCAFCSGGGHHHHHHRL	2838.18	2838.39		

Supporting Table 4. Peptides with F and their masses

Name	Peptide	Expected [M+H]+	Observed [M+H]+	Expected + BME Disulfide [M+H]+	Observed +BME Disulfide [M+H]+
Pep1	fMAFAMCSSADYKDDDDK	1940.74	1941.03	2016.73	2017.03
Pep2	fMAFAMASSADYKDDDDK	1908.76	1908.98	NA	
Pep3	fMHCIEGRFDYKDDDDK	2014.83	2015.03	2090.83	2091.09
Pep4	fMHSIEGRFDYKDDDDK	1998.85	1998.77	NA	
Pep5	fMAFAMASCSSADYKDDDDK	2098.80	2099.13	2174.80	2175.12
Pep6	fMAFAMASASCSSADYKDDDDK	2256.87	2257.13	2332.87	2233.13
Pep7	fMAFAMASASASCSSADYKDDDDK	2414.94	2415.04	2490.94	2491.06
Pep8	fMAFAMASASASASCSSADYKDDDDK	2573.01	2573.44	2649.01	2649.40

Supporting Figures

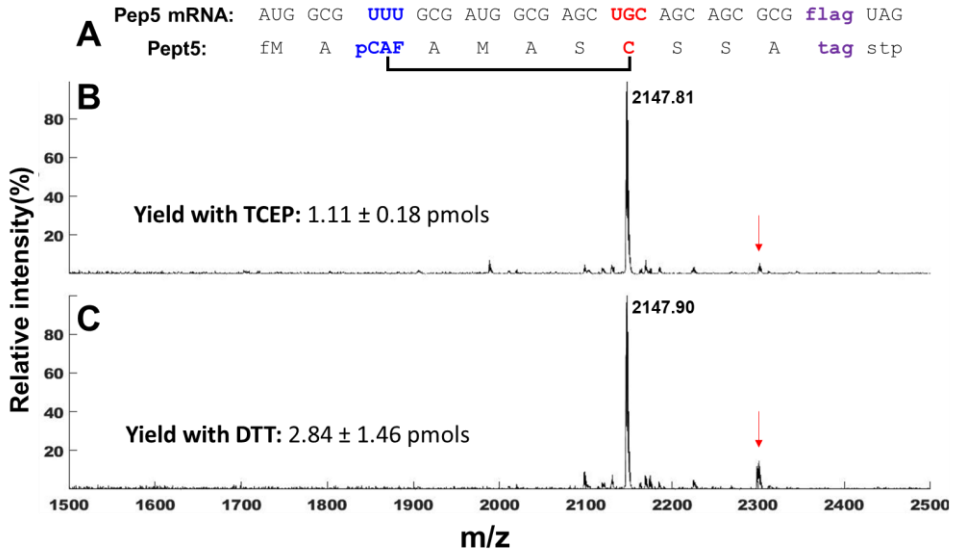


Figure S1. Comparison of TS-Solution prepared with TCEP and DTT. (A) Peptide/mRNA sequence used in the experiment. (B) MALDI MS of the translation mixture containing TCEP. The yield is for a 30 μL translation and is indicative of duplicate experiments. (C) MALDI MS of the translation mixture containing the standard DTT reducing agent. The yield is for a 30 μL translation and is indicative of duplicate experiments. The red arrows indicate the mass of the DTT adduct.

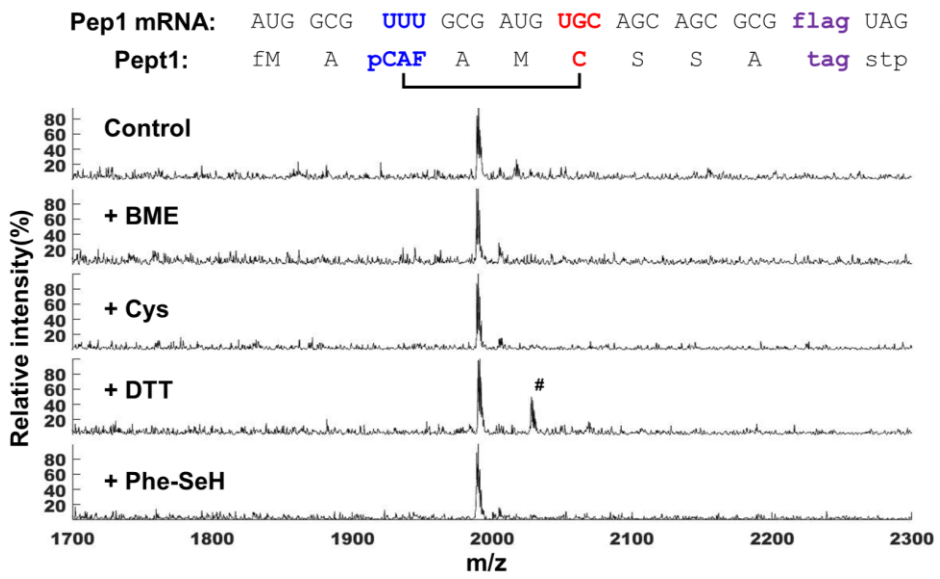


Figure S2. **Stability of PMC formed with pCAF.** Pept1 was treated with: from top to bottom, nothing (calc:1989.73,obsd.1989.25), 1mM BME (obsd.1989.98), 1mM cysteine (obsd.1989.16), 1mM DTT (obsd.1989.98) and 1mM diphenyldiselenide/10 mM ascorbic acid (obsd.1989.01) . In all cases, peptide was *in-vitro* translated, purified, and subsequently treated in a 50µL reaction scale in the presence of 50mM HEPES/KOH. Peptide was recaptured in anti-FLAG resin and measured as usual. # $[M + K]^+$ peak.

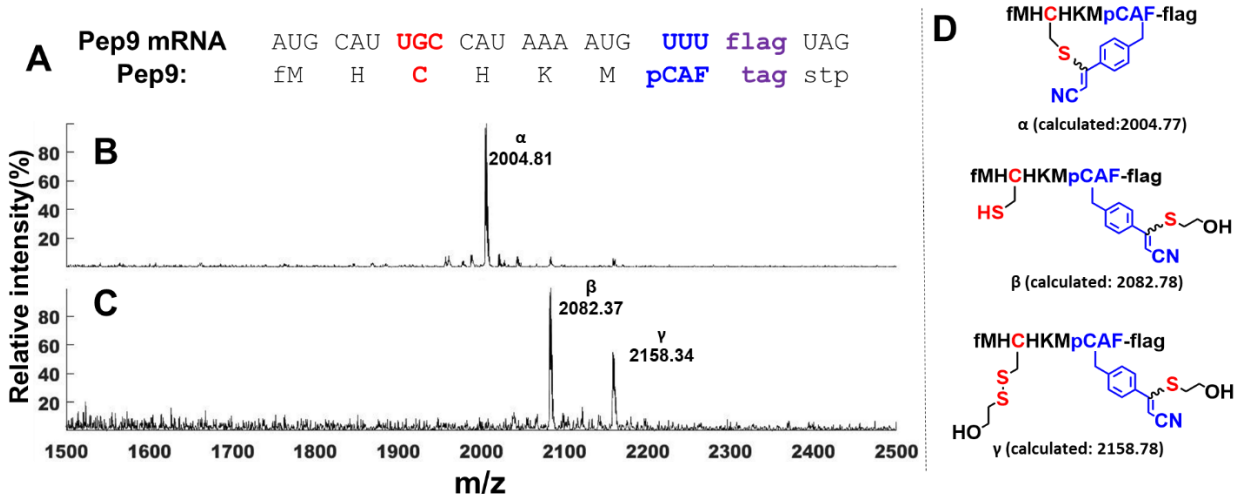


Figure S3. Intramolecular Michael-addition occurs faster than external thiol attack. (A) peptide/mRNA sequence. (B) *In-vitro* translation is carried out using free pCAF in the reaction mixture. (C) pCAF was pre-charged in the presence of 20 mM BME ensuring complete formation of BME-pCAF-$tRNA^{Phe}$. This pre-charged tRNA was added to the translation mixture. None of the cyclized peptide was observed demonstrating that thiol attack happens only after pCAF is incorporated into the peptide. (D) Structures of peptides observed in these experiments.

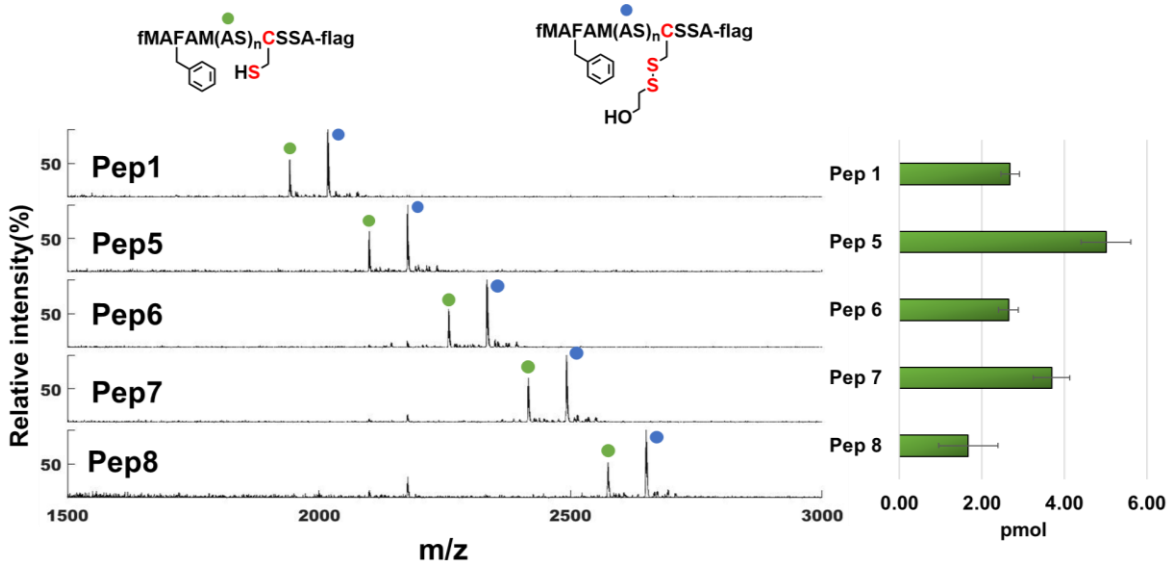
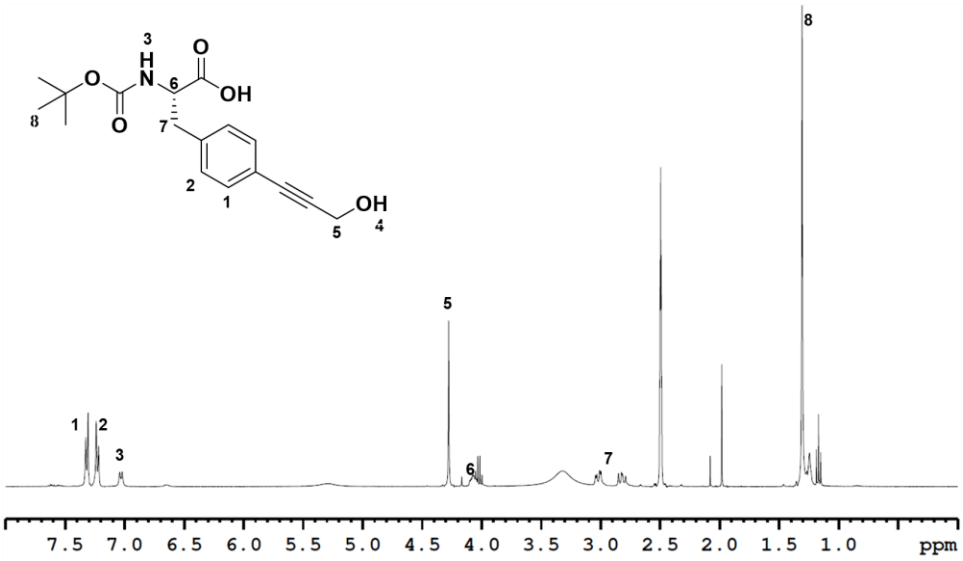
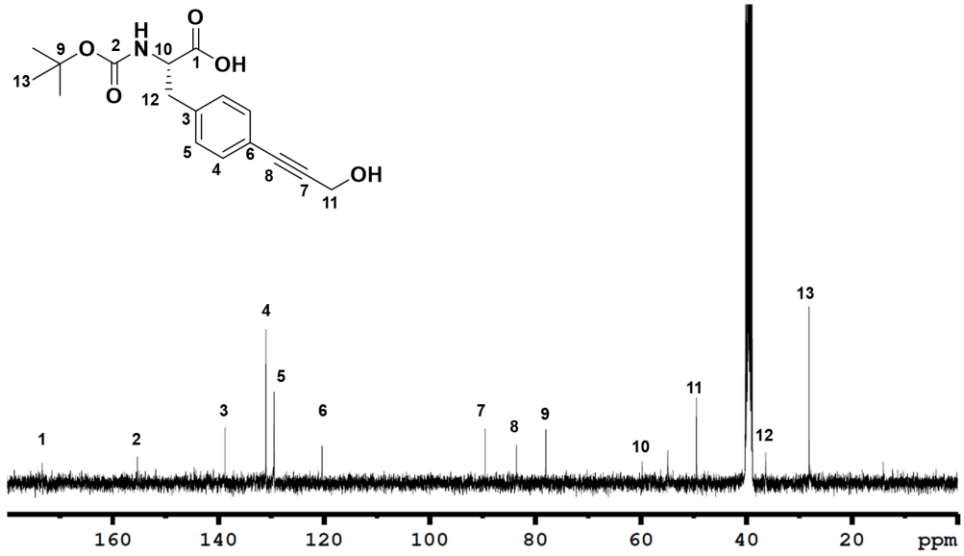


Figure S4. ***In vitro*** **translations using mRNAs described in Figure 3 but using canonical Phe instead of pCAF.** Expected and observed masses are listed in **Supporting Table 4**. The leftmost (green spot) peak indicates the intact peptide while, the rightmost (blue spot) displays a disulfide peptide formed with BME, it is important to remark that this translation was done in the presence of TCEP and residual BME from dialyzed enzymes is enough to form disulfide bonds with free Cys.

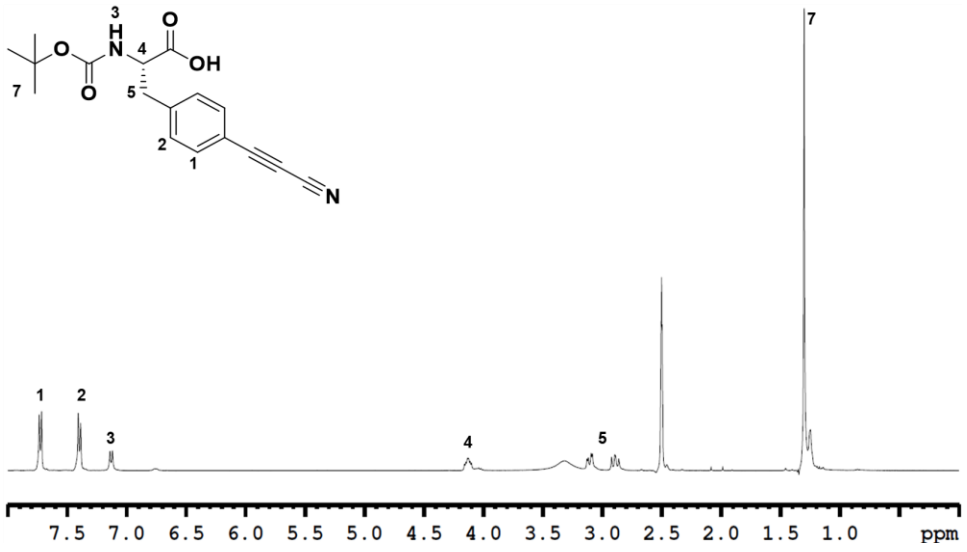
Compound NMRs



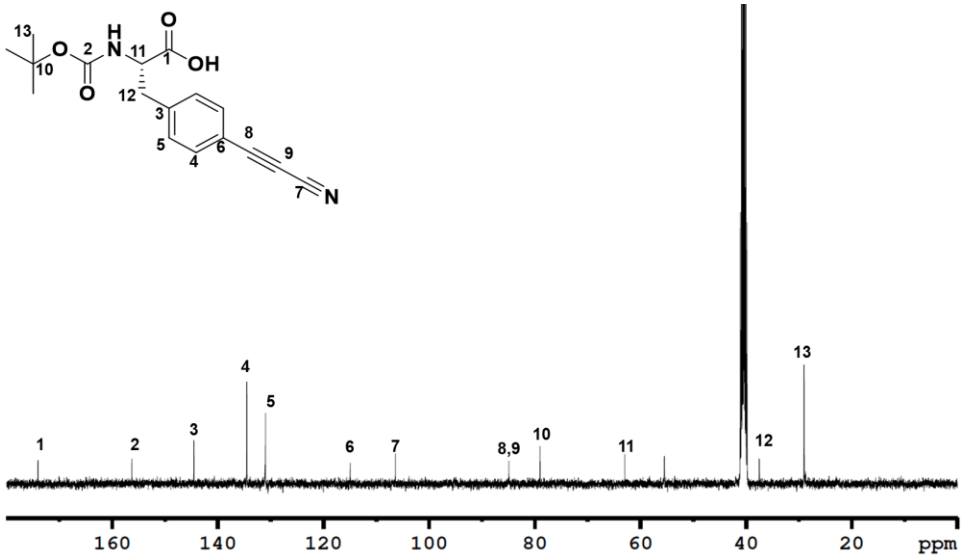
^{1}H-NMR of compound **2**



^{13}C-NMR of compound **2**



^{1}H-NMR of compound **3**



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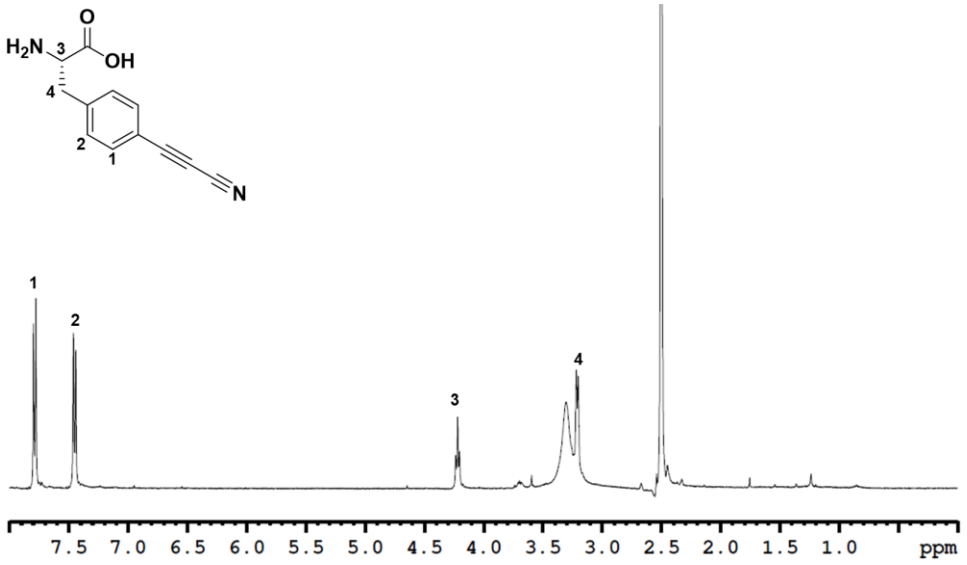
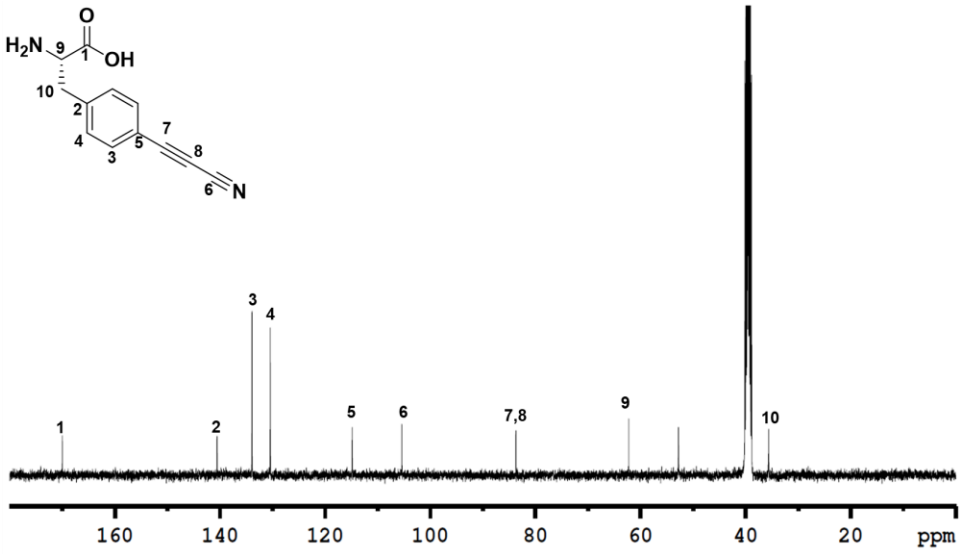


^{1}H-NMR of compound **4**



^{13}C-NMR of compound **4**

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