Supporting Information

Expanded Toolbox for Directing the Biosynthesis of Macrocyclic Peptides in Bacterial Cells

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Table S1. Oligonucleotide sequences.

Primer Number	Primer Name	Primer Sequence 5'→3'
1	TyrRS42-L32V(F)	GATGAAAAATCTGCTGTTATAGGTTTTGAACCAAG
2	TyrRS42-L32V(R)	CTTGGTTCAAAACCTATAACAGCAGATTTTTCATC
3	TyrRS42/VsFRS-L65Y(F)	GATTTGATATAATTATATACTTGGCTGATTTACACGC
4	TyrRS42/VsFRS-L65Y(R)	GCGTGTAAATCAGCCAAGTATATAATTATATCAAATC
5	TyrRS42/VsFRS-F108H-Q109G(F)	TATGTTTATGGAAGTGAACACGGCCTTGATAAGGATTATAC
6	TyrRS42/VsFRS-F108H-Q109G(R)	GTATAATCCTTATCAAGGCCGTGTTCACTTCCATAAACATA
7	TyrRS42-C159I-R162E(F)	ATGCAGGTTAATGGTATTCATTATGAAGGCGTTGATGTTGC
8	TyrRS42-C159I-R162E(R)	GCAACATCAACGCCTTCATAATGAATACCATTAACCTGCAT
9	VsFRS-L32G(F)	GATGAAAAATCTGCTGGTATAGGTTTTGAACCAAG
10	VsFRS-L32G(R)	CTTGGTTCAAAACCTATACCAGCAGATTTTTCATC
11	VsFRS-C159L-R162Q(F)	ATGCAGGTTAATGGTCTGCATTATCAGGGCGTTGATGTTGC
12	VsFRS-C159L-R162Q(R)	GCAACATCAACGCCCTGATAATGCAGACCATTAACCTGCAT
13	BglIII-SuperPrimer	GCGGATCCTACCTGACGCTTTTTATCG
14	PstI-SuperPrimer	CTATACTGCAGCGCCAAAACAGCCAAGCTGGAGAC
15	Z1C-CBD(F)	GAGGGATCCGGTTAGTGCGGCAGCAAACTGGCGGAATATGGAATTGAAGGCCGCAAAATC
16	Z2C-CBD(F)	GAGGGATCCGGTTAGACCTGCAGCAAACTGGCGGAATATGGAATTGAAGGCCGCAAAATC
17	Z3C-CBD(F)	GAGGGATCCGGTTAGACCGGCTGCAAACTGGCGGAATATGGAATTGAAGGCCGCAAAATC
18	Z4C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCTGCCTGGCGGAATATGGAATTGAAGGCCGCAAAATC
19	Z5C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCAAATGCGCGGAATATGGAATTGAAGGCCGCAAAATC
20	Z6C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCAAACTGTGCGAATATGGAATTGAAGGCCGCAAAATC
21	Z8C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCAAACTGGCGGAATGCGGAATTGAAGGCCGCAAAATC

22	Z10C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCCATTATCTGAACGCGGAATGCGGAATTGAAGGCCGCAAAATC
23	Z12C-CBD(F)	GAGGGATCCGGTTAGACCGGCAGCCATATTTATCTGACTAACGCGGAATGCGGAATTGAAGGCCGCAAAATC
24	Z15C-CBD-1(F)	GGAAGGCAGCGCGTGCGGCATTGAAGGCCGCAAAATCGAAGAAGGTAAAC
25	Z15C-CBD-2(F)	ATAGGGATCCGGCTAGACCGGCAGCCATATTTATCTGACCAACGCGGAAGGCAGCGCGTGCGGC
26	Z20C-CBD-1(F)	GCGCATATTACCCTGACCTGCGGCATTGAAGGCCGCAAAATCGAAGAAGGTAAAC
27	Z20C-CBD-2(F)	ATAGGGATCCGGCTAGACCGGCAGCCATATTTATCTGACCAACGCGGAAGGCAGCGCGCATATTACCCTGACCTGCGGC
28	Z(-6)C-CBD(F)	GAGGGATCCGGTTGCACCGGCAGCAAACTGTAGGAATATGGAATTGAAGGCCGCAAAATC
29	Z(-8)C-CBD(F)	GAGGGATCCGGTTGCACCGGCAGCAAACTGGCGGAATAGGGAATTGAAGGCCGCAAAATC
30	CBD(R)	TAGTCCTCGAGCAGTTCGAGACCGTTGTTACCGTTGT

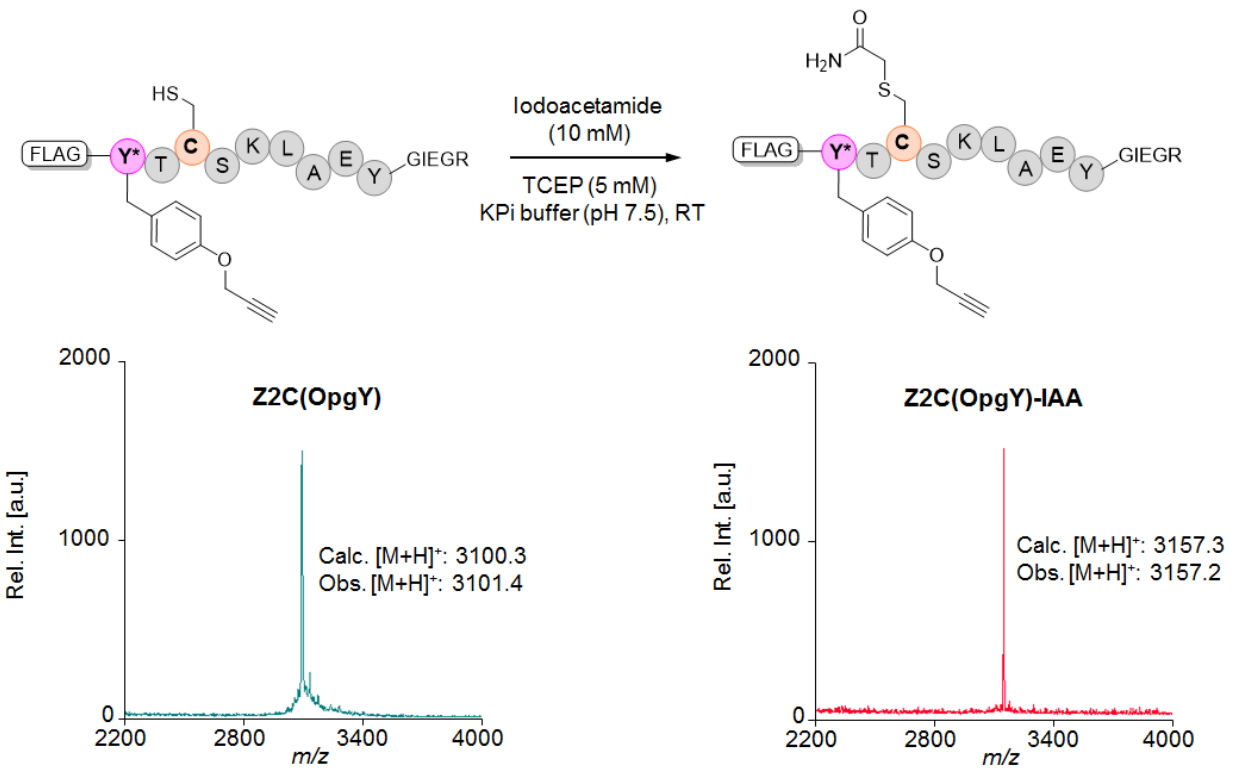


Figure S1. Iodoacetamide alkylation reaction for detection of free thiols. MALDI-TOF MS spectra of Z2C(OpgY) before and after incubation with iodoacetamide, indicating quantitative alkylation of the free cysteine. Y* = O-propargyl-tyrosine (OpgY).

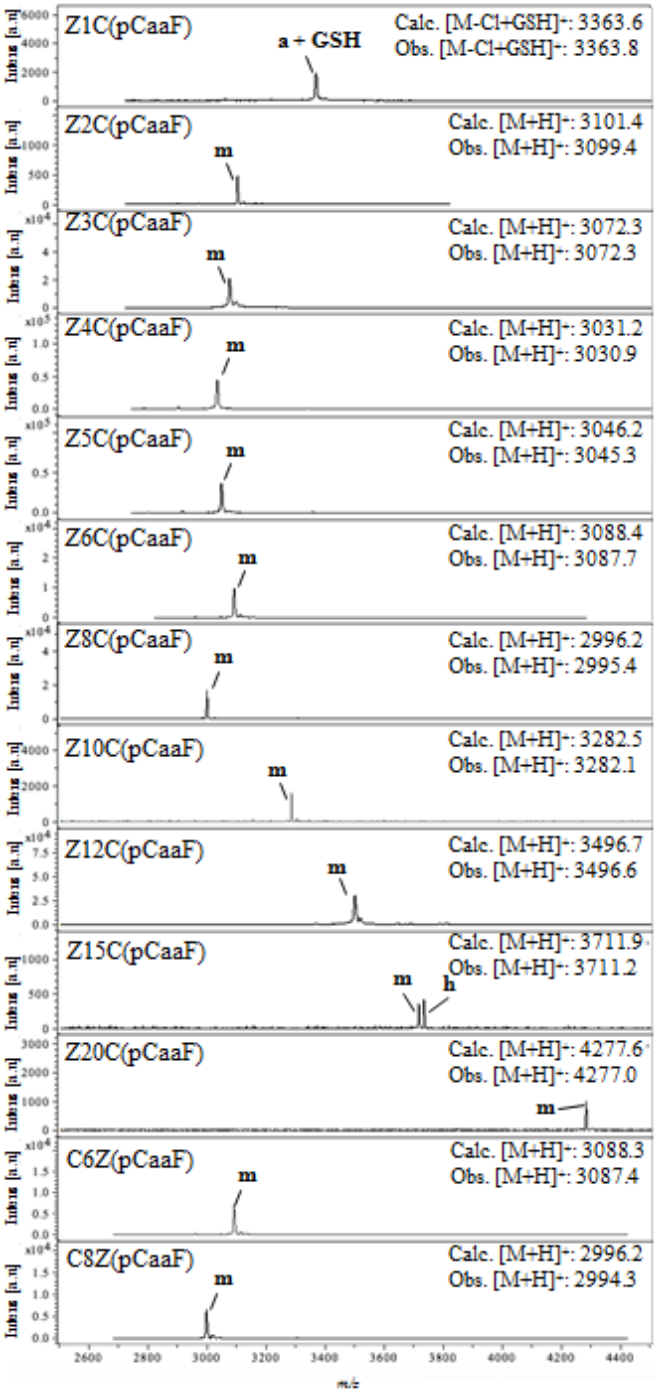


Figure S2. MALDI-TOF MS spectra corresponding to purified pCaaF-containing macrocyclic peptides after proteolytic cleavage of the CBD tag. Calculated and observed *m/z* values corresponding to the proton adduct of the macrocyclic peptide ('m') are indicated. 'h' = hydrolysis product; 'a+GSH' = alkylated glutathione adduct.

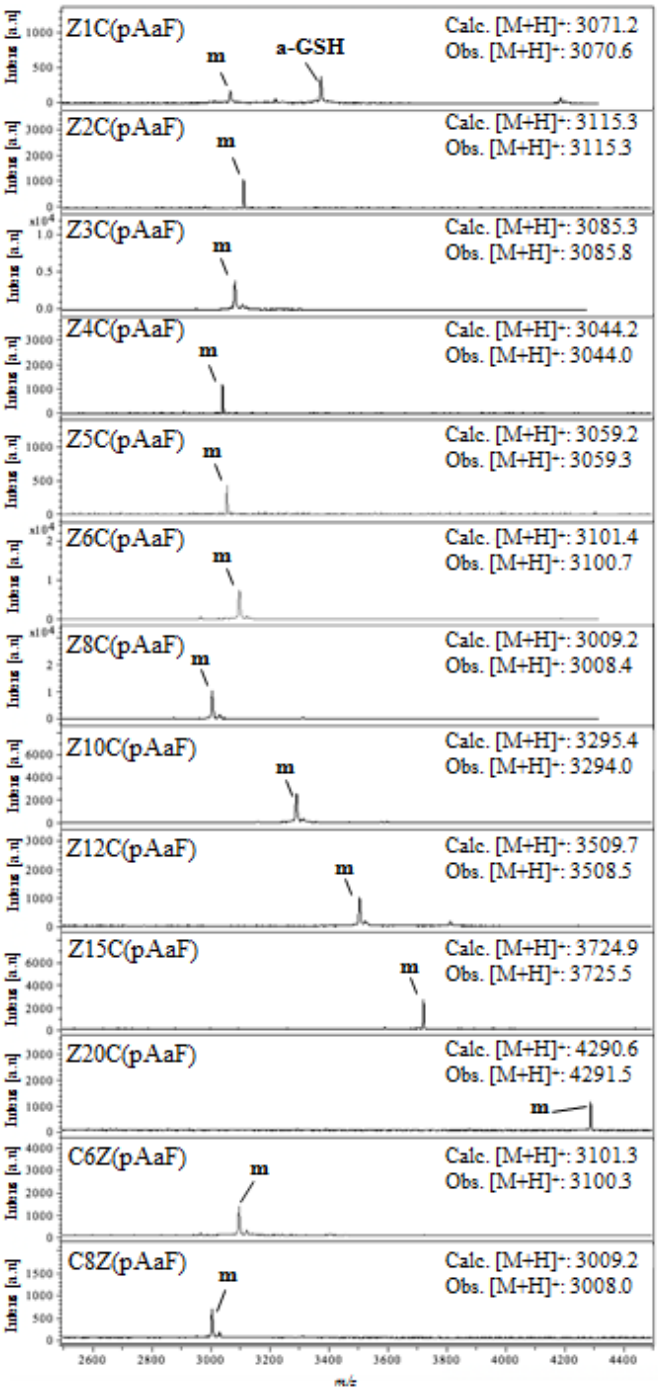


Figure S3. MALDI-TOF MS spectra corresponding to purified pAaF-containing macrocyclic peptides after proteolytic cleavage of the CBD tag and treatment with iodoacetamide. Calculated and observed *m/z* values corresponding to the proton adduct of the macrocyclic peptide ('m') are indicated. 'h' = hydrolysis product; 'a+GSH' = alkylated glutathione adduct.

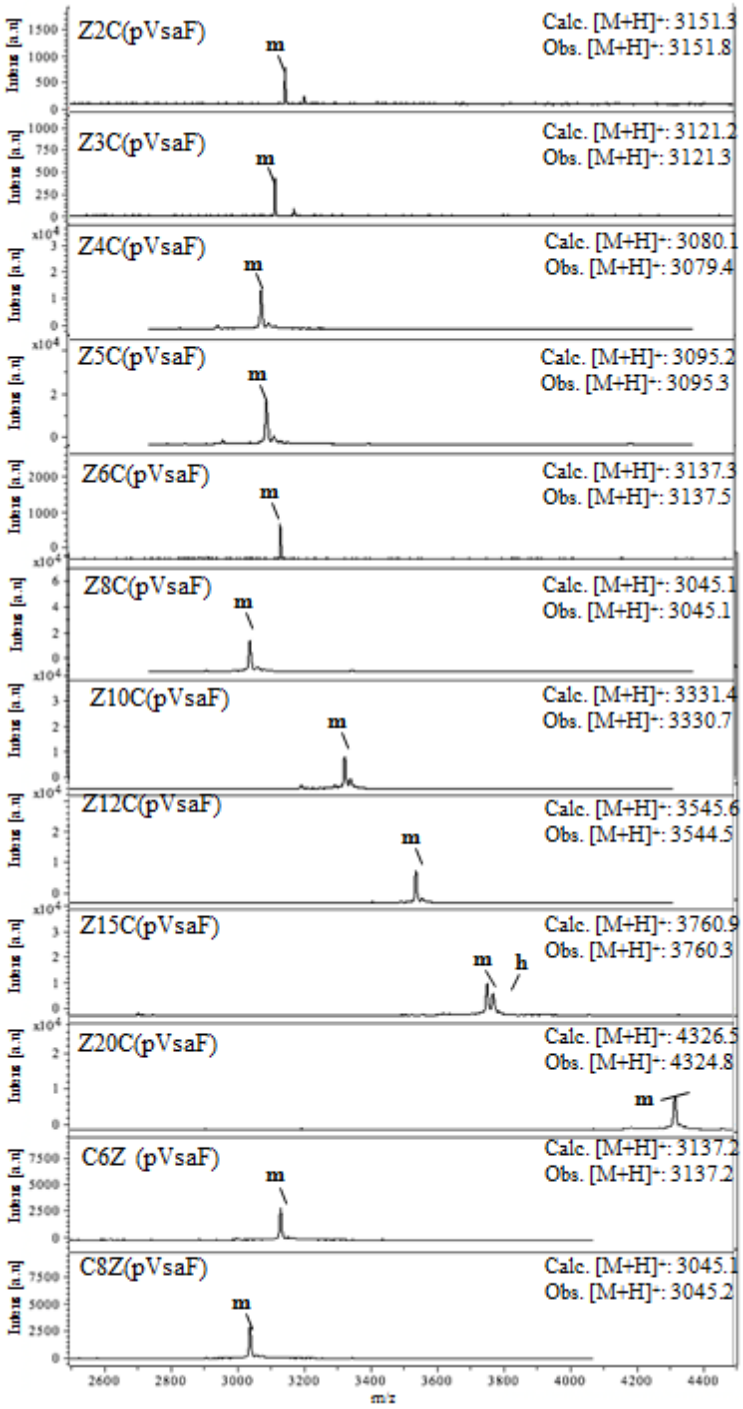


Figure S4. MALDI-TOF MS spectra corresponding to purified pVsaF-containing macrocyclic peptides after proteolytic cleavage of the CBD tag and treatment with iodoacetamide. Calculated and observed *m/z* values corresponding to the proton adduct of the macrocyclic peptide ('m') are indicated. 'h' = water adduct.

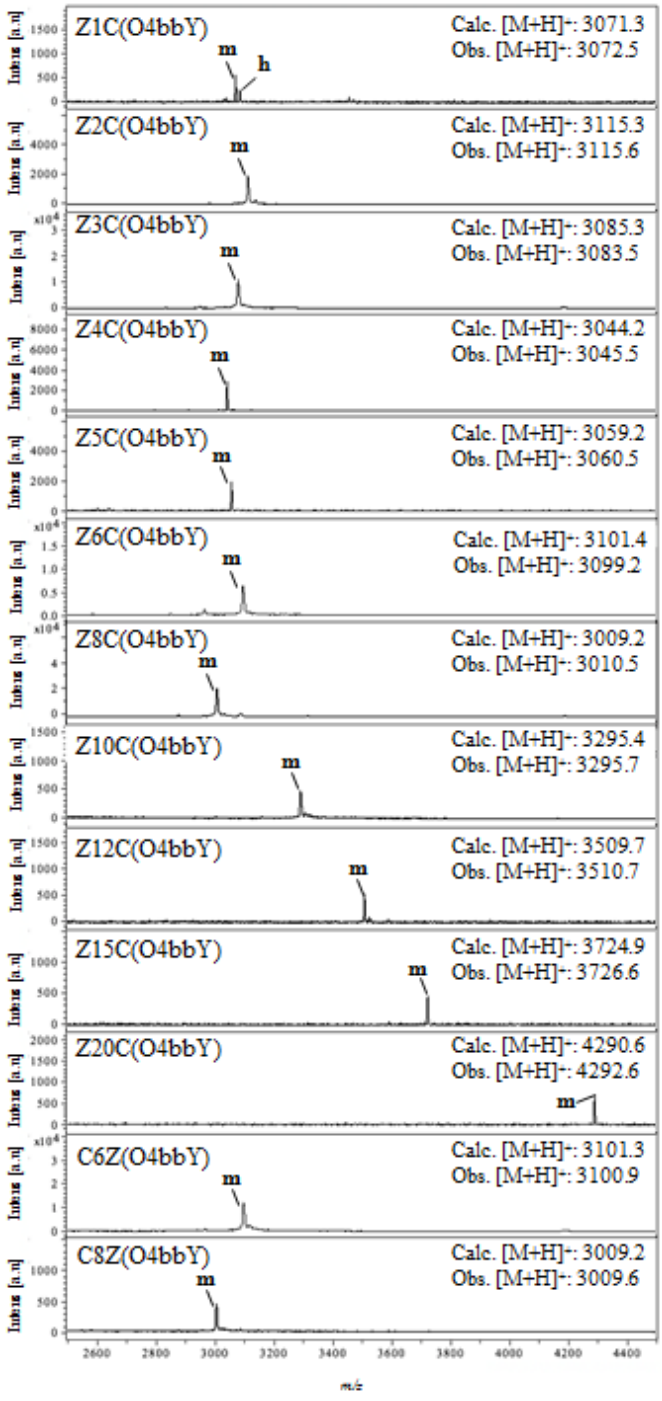


Figure S5. MALDI-TOF MS spectra corresponding to purified O4bbY-containing macrocyclic peptides after proteolytic cleavage of the CBD tag. Calculated and observed *m/z* values corresponding to the proton adduct of the macrocyclic peptide ('m') are indicated. 'h' = hydrolysis product.

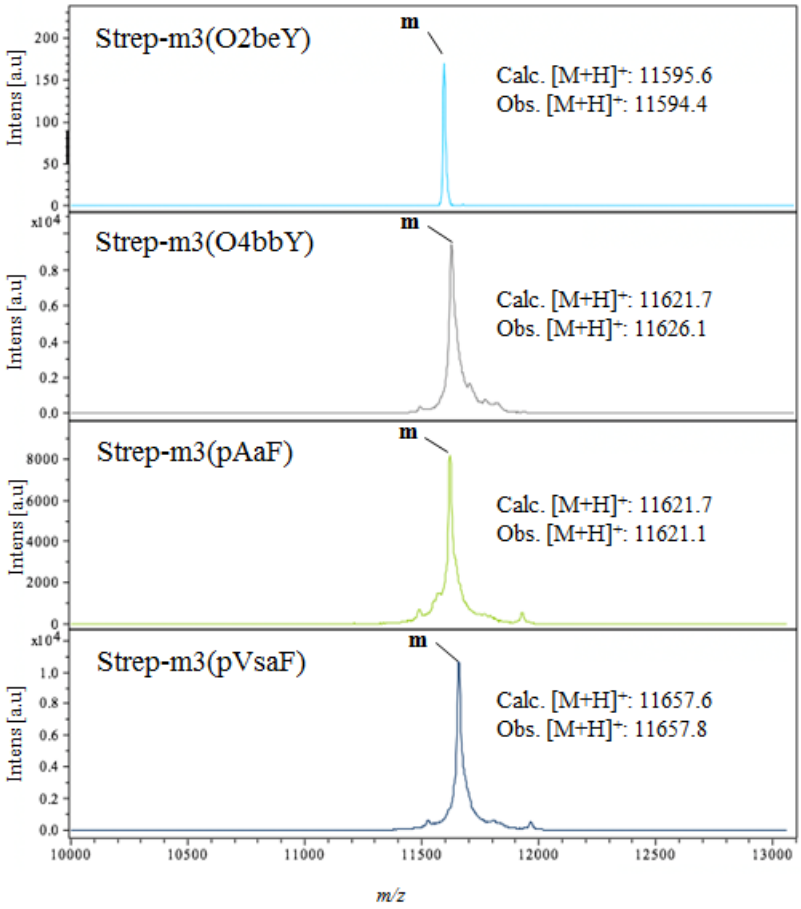


Figure S6. MALDI-TOF MS spectra corresponding to the purified Strep-m3 derivatives containing different eUAAs. Calculated and observed *m/z* values corresponding to the proton adduct of the CBD-fused macrocycle are indicated. The pAaF- and pVsaF-containing constructs were treated with iodoacetamide prior to MS analysis.

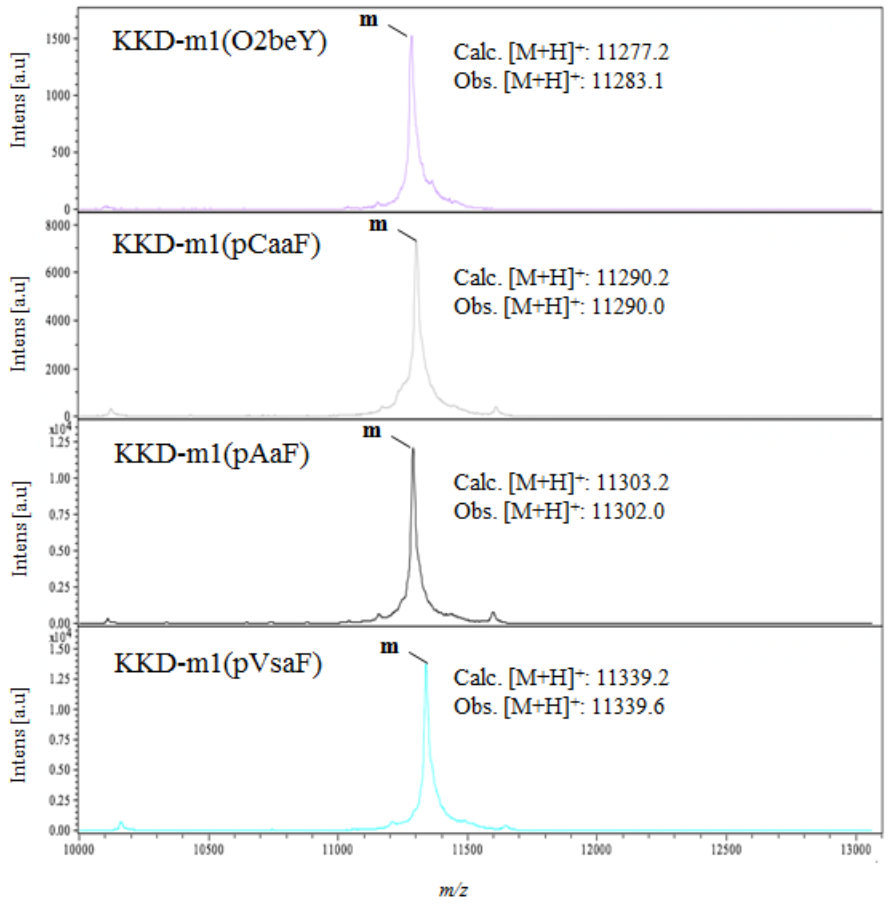
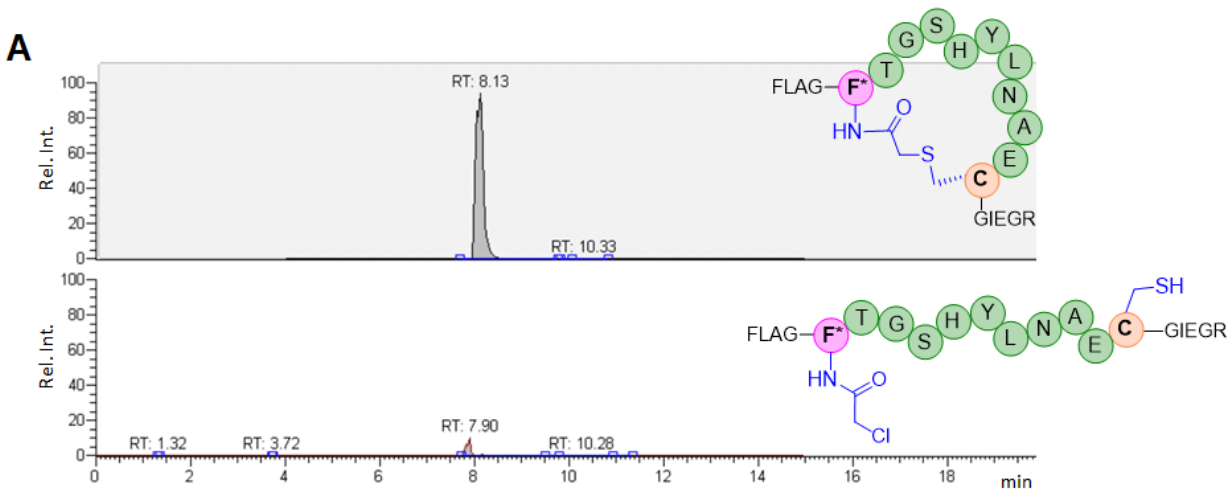


Figure S7. MALDI-TOF MS spectra corresponding to the purified KKD-m1 derivatives containing different eUAAs. Calculated and observed *m/z* values corresponding to the proton adduct of the CBD-fused macrocycle are indicated. The pAaF- and pVsaF-containing constructs were treated with iodoacetamide prior to MS analysis.



Construct	Macrocycle Peak Area	Linear peptide Peak Area
Z10C(pCaaF)	219,789,751	15,656,114

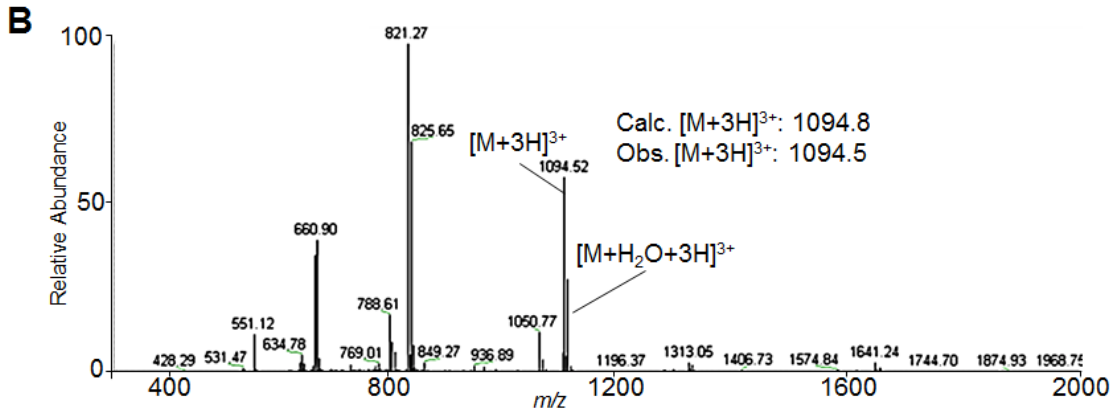
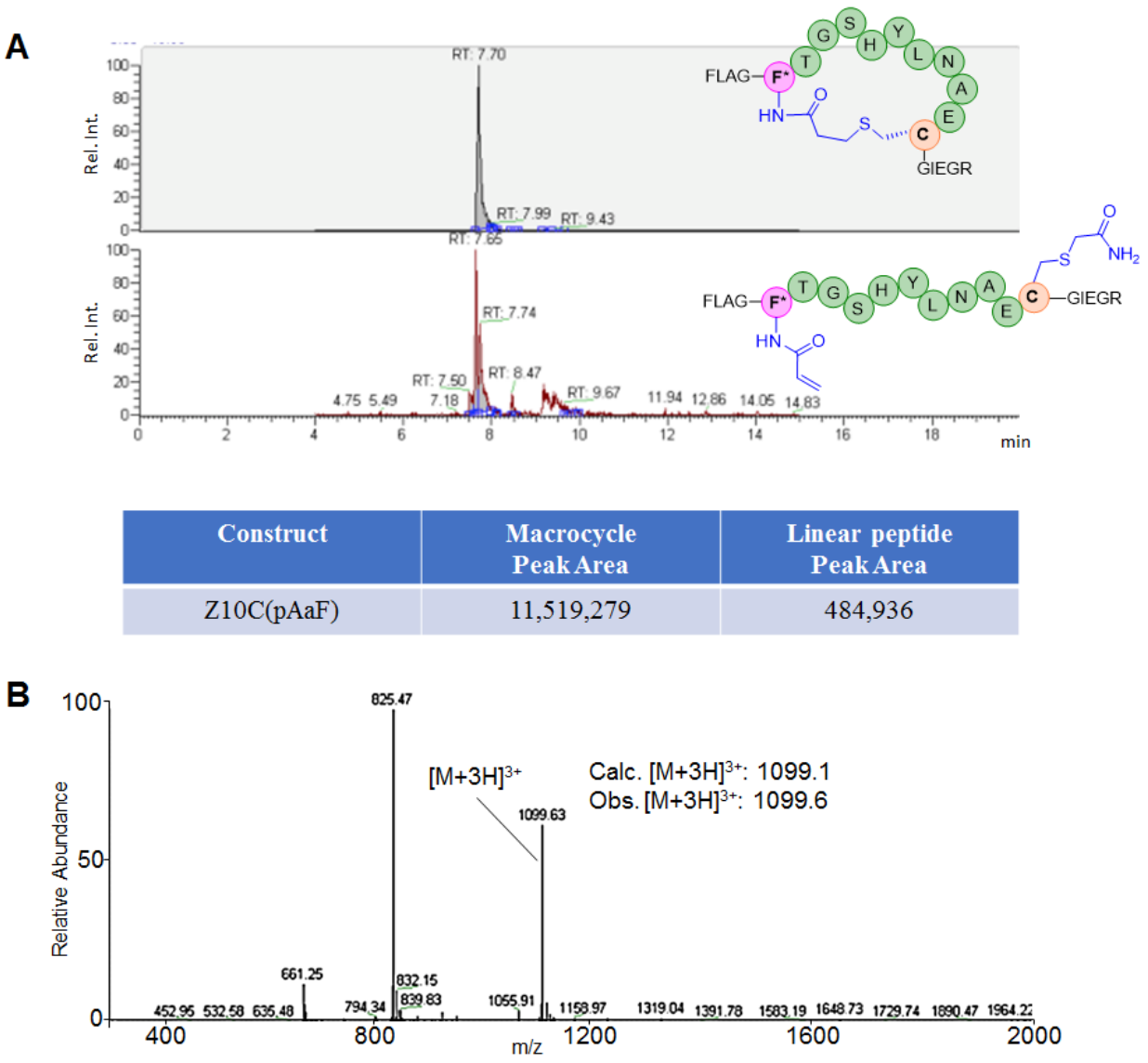
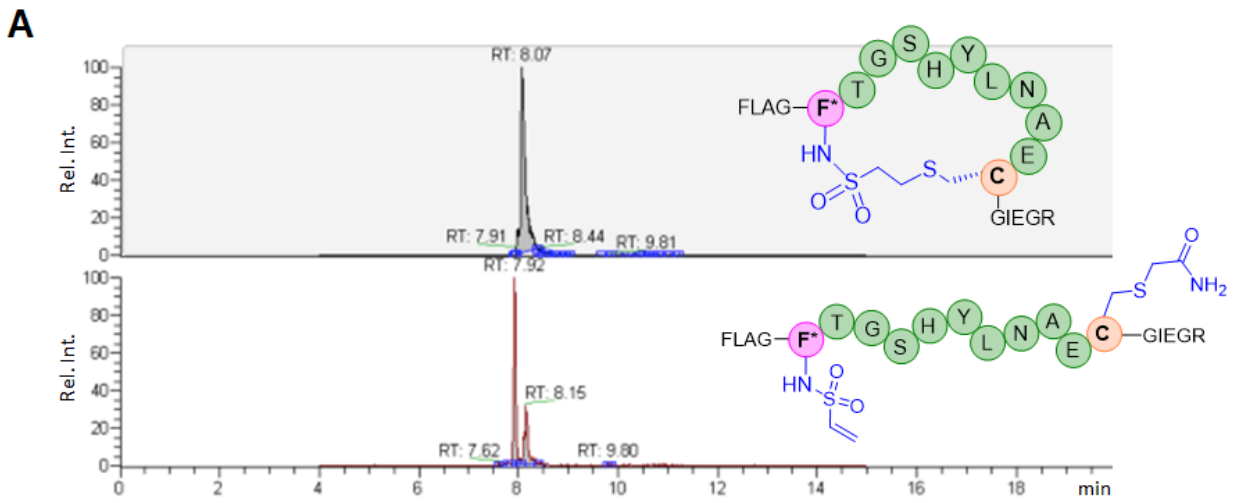


Figure S8. LC-MS analysis of Z10C(pCaaF) peptide. (A) LC-MS extracted-ion chromatogram corresponding to the macrocyclic product (*top*) and acyclic peptide (*bottom*). (B) MS/MS spectrum of the macrocyclic Z10C(pCaaF) peptide. F* = *para*-substituted Phe.



Construct	Macrocycle Peak Area	Linear peptide Peak Area
Z10C(pAaF)	11,519,279	484,936

Figure S9. LC-MS analysis of Z10C(pAaF) peptide. (A) LC-MS extracted-ion chromatogram corresponding to the macrocyclic product (*top*) and iodoacetamide-alkylated acyclic peptide (*bottom*). (B) MS/MS spectrum of the macrocyclic Z10C(pAaF) peptide. F* = *para*-substituted Phe.



Construct	Macrocycle Peak Area	Linear peptide Peak Area
Z10C(pVsaF)	7,946,180	254,443

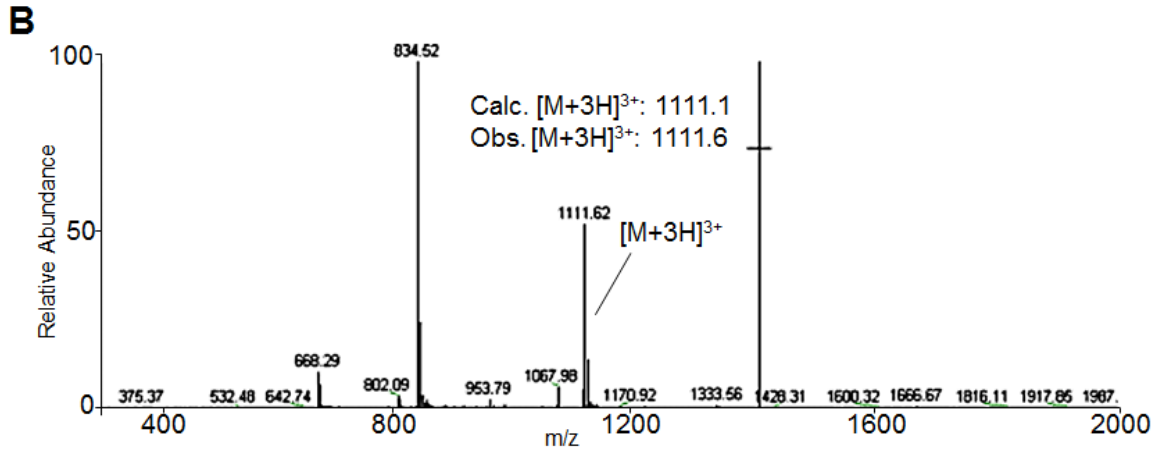
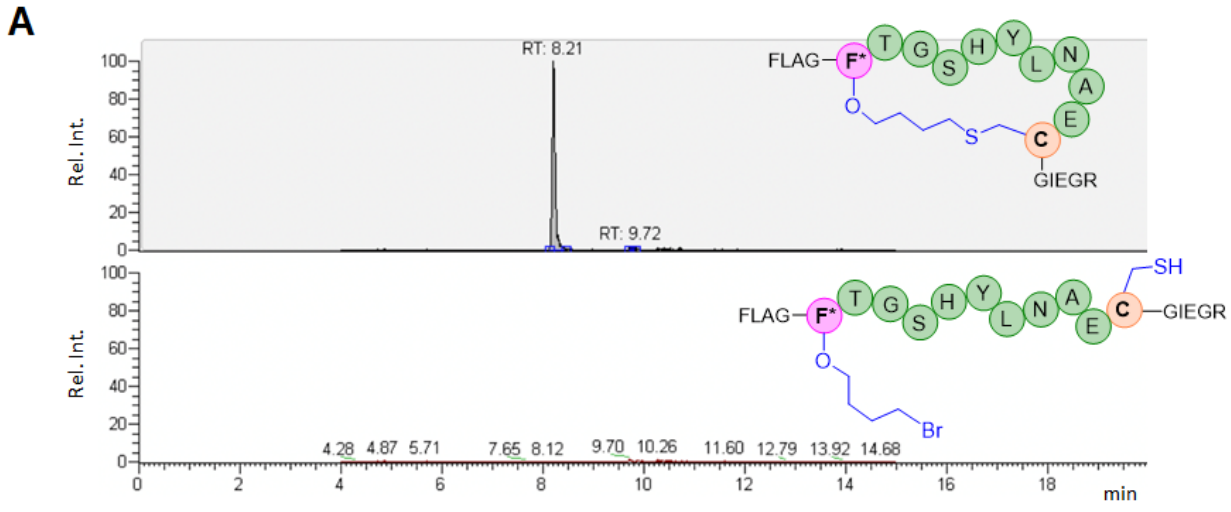


Figure S10. LC-MS analysis of Z10C(pVsaF) peptide. (A) LC-MS extracted-ion chromatogram corresponding to the macrocyclic product (*top*) and iodoacetamide-alkylated acyclic peptide (*bottom*). (B) MS/MS spectrum of the macrocyclic Z10C(pVsaF) peptide. F* = *para*-substituted Phe.



Construct	Macrocycle Peak Area	Linear peptide Peak Area
Z10C(O4bbY)	450,661	3,176

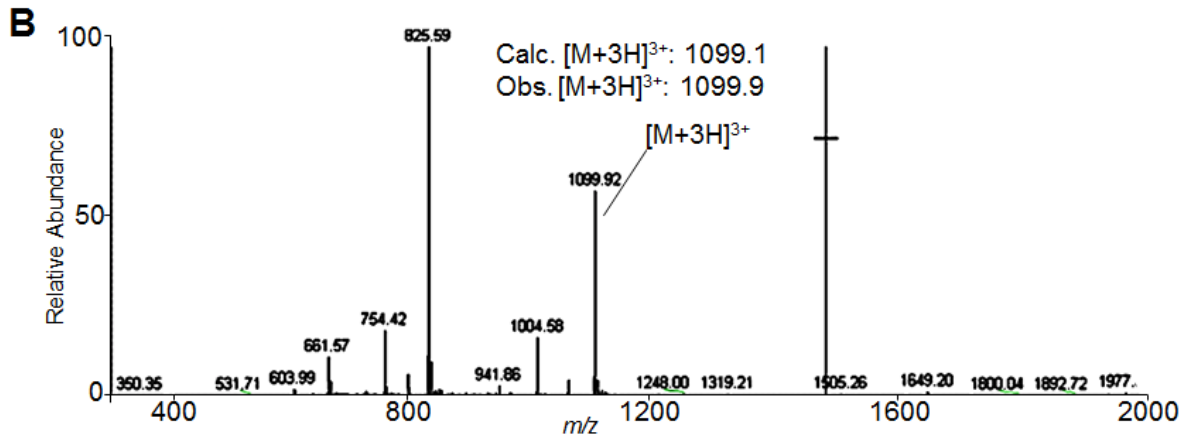


Figure S11. LC-MS analysis of Z10C(O4bbY) peptide. (A) LC-MS extracted-ion chromatogram corresponding to the macrocyclic product (*top*) and acyclic peptide (*bottom*). (B) MS/MS spectrum of the macrocyclic Z10C(O4bbY) peptide. F* = *para*-substituted Phe.

Experimental Procedures

General Information. Chemical reagents and solvents were purchased from Sigma-Aldrich, Acros Organics, and Chem-Impex. Silica gel chromatography purifications were carried out by using AMD Silica Gel 60 230-400 mesh. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance spectrometers by using solvent peaks as the reference. LC-MS analyses were performed on a Thermo-Scientific LTQ Velos ESI/ion-trap mass spectrometer coupled to an Accela U-HPLC. Peptides were analyzed using Agilent poroshell 120 C18 column (particle size 2.7 μm, 100 x 4.6 mm) and a linear gradient 5% to 95% ACN (with 0.1% formic acid) in water (with 0.1% formic acid) over 10 min. MALDI-TOF spectra were acquired on a Bruker Autoflex III MALDI-TOF spectrometer using α–cyano-4-hydroxycinnamic acid as the matrix.

Cloning and Plasmid Construction. The AARS enzymes for incorporation of pVsaF and pAaF were prepared according to a previous report.[1] Mutations were introduced via site-directed mutagenesis using primers 1-8 and 13-14 for pAaF-RS and primers 9-14 for pVsaF-RS and using the pEVOL-*p*-acetyl phenylalanine synthetase (pEVOL-pAcF-RS)[2] as the template. Genes encoding for each of the synthetases were placed under an arabinose-inducible promoter in a pEVOL vector. The PCR product was cloned into the *Pst* I/*Bgl* II cassette of pEVOL-pAcF-RS. Genes encoding for the precursor proteins were placed under an IPTG-inducible T7 promoter in a pET22 vector. Constructs were prepared through PCR amplification with forward primers 15-29 and reverse primer 30 using pET22_FLAG_CBD as the template. The PCR product was cloned into the *BamH* I/*Xho* I cassette of pET22_FLAG_CBD. The preparation of plasmids for the expression of the streptavidin and Keap1 binding proteins was described previously.[3] The

recombinant DNA constructs were transformed into *E. coli* DH5α cells and selected on LB agar plates supplemented with ampicillin (100 mg L^{-1}) for pET22-based constructs and chloramphenicol (34 mg L^{-1}) for pEVOL-based constructs. The correct transformants were identified by Sanger sequencing.

Fluorescence-based Assay for Measurement of ncAA Incorporation Efficiency. *E. coli* BL21(DE3) cells were co-transformed with a pET22_YFP(stop) plasmid encoding MetGly-(amber stop)-YFP-His_6[4] and pEVOL plasmids containing various aminoacyl-tRNA synthetases. Cells were grown in LB media supplemented with ampicillin (100 mg L^{-1}) and chloramphenicol (34 mg L^{-1}) at 37 °C overnight. The overnight cultures were then used to inoculate 96-deep well plates containing M9 media. Cultures were grown to an OD_{600} of 0.6 and induced with L-arabinose (0.06%), IPTG (1mM), and the respective ncAA (2 mM). After overnight growth at 27 °C, the cell cultures were diluted (1:1) with potassium phosphate (Kpi) buffer (50 mM, NaCl 150 mM, pH 7.5), and fluorescence intensity (λ_{ex} = 514 nm; λ_{em} = 527 nm) was determined using a Tecan Infinite 1000 plate reader. Cell cultures containing no ncAA were used as controls. Each sample was measured in triplicate.

Protein Expression and Purification. Proteins were expressed in *E. coli* BL21(DE3) cells co-transformed with the plasmid encoding the biosynthetic precursor and the pEVOL vector containing the appropriate aminoacyl-tRNA synthetase and cognate suppressor tRNA. After overnight growth, cells were used to inoculate M9 media supplemented with ampicillin (100 mg L^{-1}), chloramphenicol (34 mg L^{-1}), and 1% glycerol. Cells were grown to an OD_{600} of 0.5-0.6, at which time protein expression was induced by adding L-arabinose (0.06%), IPTG (1 mM), and

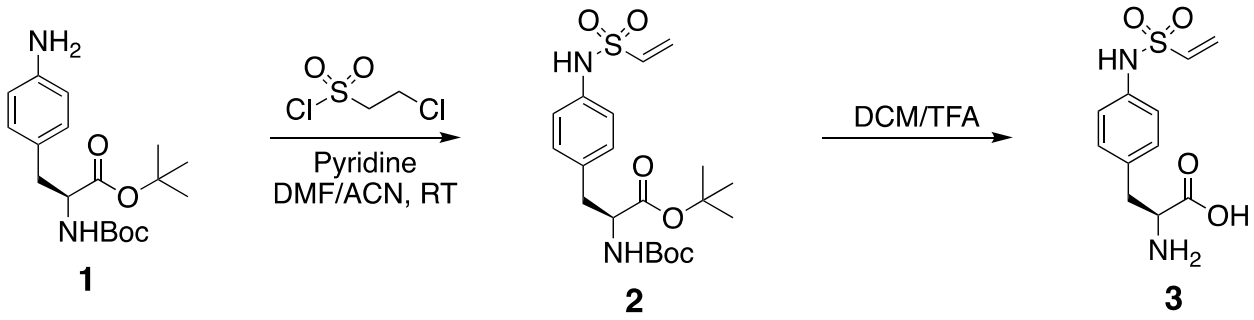
ncAA (2 mM). Cultures were grown at 27 °C for 18h. Cells were harvested by centrifugation at 4000x g. Media was removed and cells were resuspended in Tris buffer (50 mM; pH 7.5) containing 300 mM NaCl, 20 mM imidazole and lysed by sonication. Protein purification by Ni-NTA affinity chromatography was carried out using Tris buffer (50 mM; pH 7.5) containing either 50 mM imidazole and 300 mM NaCl or 300 mM imidazole and 150 mM NaCl for protein loading and elution, respectively. Eluted protein was concentrated down in potassium phosphate (Kpi) buffer (50 mM, NaCl 150 mM, pH 7.5) using an Amicon 10 kDa spin filter and placed at -80 °C for long term storage. Removal of the CBD purification tag was achieved through incubation of the protein with Factor Xa protease (NEB) following the manufacturer's protocol overnight at room temperature. Protein cleavage was monitored via MALDI-TOF MS analysis. The resulting reaction was acidified with TFA (0.1%) and purified via solid-phase extraction with a step gradient of acetonitrile in water (+ 0.1% TFA). Fractions containing the peptide were confirmed via MALDI-TOF MS and lyophilized. OpgY-containing constructs were prepared using a OpgY-specific AARS as described previously.[5] Keap1 Kelch Domain was expressed and purified as described previously.[3]

Iodoacetamide Alkylation. For the pVsaF- and pAaF-containing constructs, after Factor Xa cleavage, the peptide were diluted to a final concentration of 250 μM in potassium phosphate (Kpi) buffer (50 mM, NaCl 150 mM, pH 7.5). To the reaction was added tris(2-carboxyethyl) phosphine (TCEP) (5mM), iodoacetamide (10 mM), and the pH was adjusted to 8.5. The reaction was allowed to incubate for 2 hours at room temperature and the occurrence of alkylation was determined by MALDI-TOF MS. The reaction mixtures were analyzed directly by LC-MS under the conditions specified in the General Information section. Cyclization efficiency was quantified by integrating

the area under the peak from extracted-ion chromatograms (TIC) corresponding to the doubly and triply charged ions of unmodified peptide (= cyclic) and alkylated adduct (= linear).

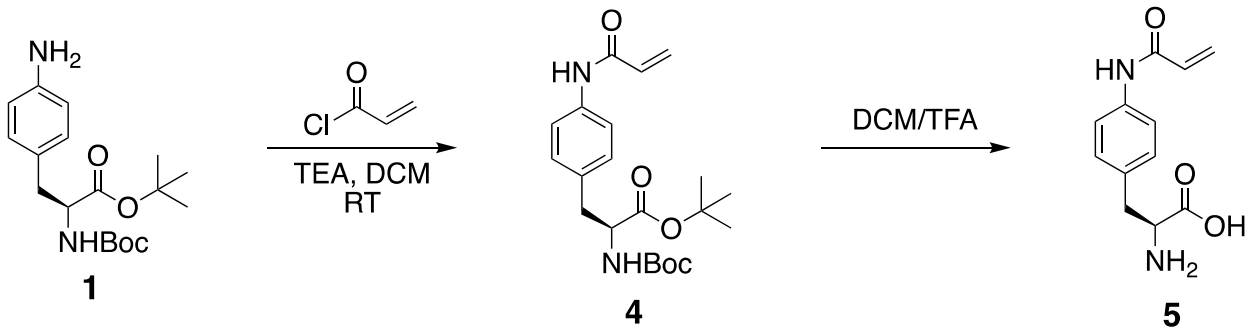
Binding Assays. Keap1 was immobilized on 96-well microtiter plates by incubating each well with 100 µL of a 4 µM protein solution in PBS buffer (10 mM Na_2HPO_4, 1.8 mM, KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) overnight at 4 °C. Protein was removed and the plates were blocked with 0.5% bovine serum albumin in PBS buffer for 2h at room temperature. Pre-blocked Streptavidin coated plates were purchased from Sigma-Aldrich (Ca. No. S6940). Each well was incubated for 1h at room temperature with 100 µL of FLAG-fused peptide with 1:2 serial dilutions down the plate. The plate was washed with 3X 150 µL wash buffer (0.05% tween-20 in PBS buffer) and incubated with 100 µL of 1:2500 dilution of HRP-conjugate mouse anti-FLAG polyclonal antibody (Sigma-Aldrich) for 1h at room temperature. After washing 3x with wash buffer, 100 µL of 2.2 mM o-phenylenediamine dihydrochloride, 4.2 mM urea hydrogen peroxide, 100 mM dibasic sodium phosphate, and 50 mM sodium citrate, pH 5.0, were added to each well, followed by measurement of the absorbance at 450 nm after 10–20 min using a Tecan Infinite 1000 plate reader. Equilibrium dissociation constants (K_D) were determined by fitting the dose–response curves to a 1:1 binding isotherm equation via nonlinear regression using SigmaPlot. Mean values and standard deviations were calculated from experiments performed in duplicate.

Synthetic Procedures:



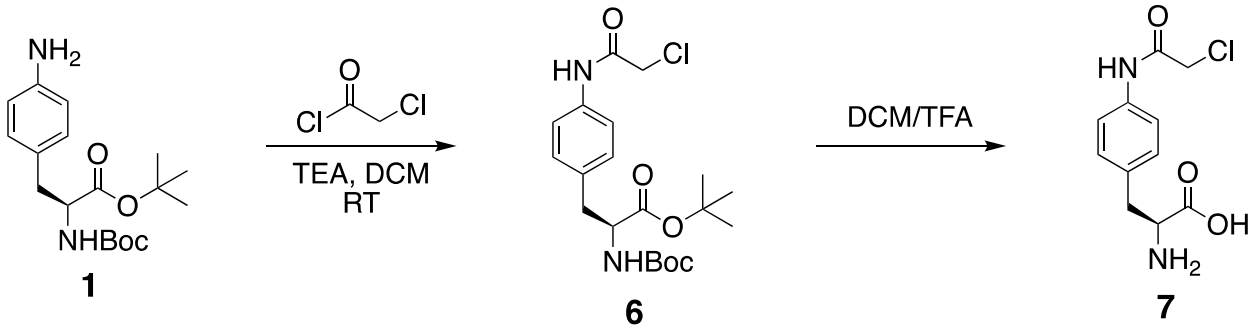
Synthesis of *p*-vinylsulfonylamide phenylalanine (pVsaF): pVsaF (**3**) was synthesized according to a modified version of a reported procedure.[1] N-Boc-O-*t*-Bu-4-aminophenylalanine (**1**) (0.7 g, 2.1 mmol) was dissolved in a 1:1 mixture of DMF/ACN (7 mL total). To the reaction flask was added pyridine (0.5 mL, 6.3 mmol, 3.0 equiv), followed by the dropwise addition of 2-chloroethanesulfonyl chloride (0.3 mL, 3.1 mmol, 1.5 equiv). The reaction was allowed to stir at RT for 3h and was quenched with 5% HCl (20 mL). The reaction mixture was transferred to a separatory funnel and extracted with EtOAc (20 mL) and the phases were separated. The aqueous phase was adjusted to neutral pH and was extracted with 2 X 20 mL of EtOAc. Organic layers were pooled, washed with brine and dried over Na_2SO_4. Solvent was removed via rotary evaporation to yield a crude orangish-yellow solid. Crude was adsorbed onto silica gel and purified via flash chromatography (10:3 Hex:EtOAc) to afford N-Boc-O-*t*-Bu-4-vinylsulfonylamide phenylalanine (**2**) as a orangish-yellow oil. The purified product was deprotected in a 1:1 mixture of DCM:TFA (10 mL) for 5h at RT. Volatiles were removed by rotary evaporation. The reaction mixture was taken up in Et_2O (20 mL) and removed via rotary evaporation. This process was repeated 5X to remove residual TFA. The deprotected product was taken up in 10 mL of 50mM HCl, flash frozen, and lyophilized to afford **3** as an orange solid (0.14g, 25% yield). ^{1}H NMR (500

MHz, MeOD) δ = 7.04 (d, *J*= 5.0 Hz, 2H), 6.99 (d, *J*= 5.0 Hz, 2H), 6.46 (dd, *J*= 15.0, 10.0 Hz, 1H), 5.95 (d, *J*= 15.0 Hz, 1H), 5.75 (d, *J*= 10.0 Hz, 1H), 4.02 (t, *J* = 6.8 Hz, 1H), 3.08 (dd, *J* = 21.5, 9.0 Hz, 1H), 2.91 (dd, *J* = 13.5, 7.0 Hz, 1H); ^{13}C NMR (126 MHz, MeOD) δ= 138.78, 137.36, 132.50, 131.52, 128.06, 124.69, 122.23, 55.2, 36.78; MS (ESI): calcd for $C_{11}H_{14}N_2O_4S$: 271.32 $[M+H]^+$; found: 271.46



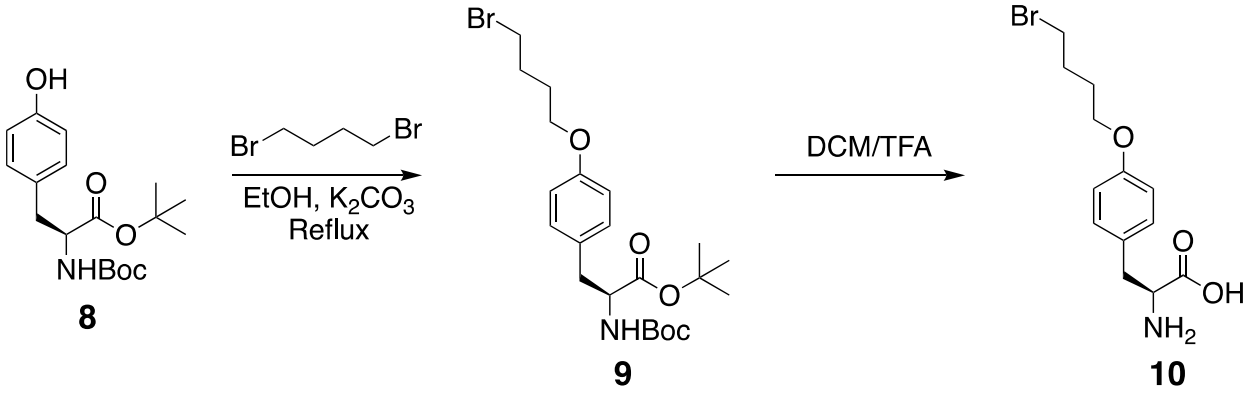
Synthesis of *p*-acrylamide phenylalanine (pAaF): Synthesis of pAaF (**5**) was adapted from a previously reported protocol.[1] N-Boc-O-*t*-Bu-4-aminophenylalanine (**1**) (0.6 g, 1.8 mmol) was dissolved in dichloromethane (6 mL). To the reaction flask was added triethylamine (0.7 mL, 5.3 mmol, 3.0 equiv), followed by the dropwise addition of acryloyl chloride (0.2 mL, 2.7 mmol, 1.5 equiv). The reaction was allowed to stir at RT for 3h and solvent was removed via rotary evaporation. The reaction mixture was taken up in EtOAc (20 mL) and transferred to a separatory funnel and the reaction was extracted with ddH_2O (30 mL). The aqueous phase was extracted with 3 X 20 mL of EtOAc. Organic layers were pooled, washed with brine and dried over Na_2SO_4. Solvent was removed via rotary evaporation to yield a crude yellow solid. Crude was adsorbed onto silica gel and purified via flash chromatography (10:3 Hex:EtOAc) to afford

N-Boc-O-*t*-Bu-4-acrylamide phenylalanine (**4**) as a yellow oil. The purified product was deprotected in a 1:1 mixture of DCM:TFA (10 mL) for 5h at RT. Volatiles were removed by rotary evaporation. The reaction mixture was taken up in Et_2O (20 mL) and removed via rotary evaporation. This process was repeated 5X to remove residual TFA. The deprotected product was taken up in 10 mL of 50mM HCl, flash frozen, and lyophilized to afford **5** as a brown solid (0.11g, 27% yield). ^{1}H NMR (400 MHz, MeOD) δ = 7.51 (d, *J* = 8.2 Hz, 2H), 7.15 (d, *J* = 8.3 Hz, 2H), 6.41 – 6.13 (m, 2H), 5.65 (d, *J* = 9.8 Hz, 1H), 4.11 (t, *J* = 6.8 Hz 1H), 3.16 (dd, *J* = 11.6, 5.2 Hz, 1H), 3.01 (dd, J = 14.5, 7.6 Hz, 1H); ^{13}C NMR (126 MHz, MeOD) δ= 170.57, 165.59, 138.71, 131.71, 130.83, 130.32, 127.33, 121.32, 54.47, 36.10; MS (ESI): calcd for $C_{12}H_{14}N_2O_3$: 235.11 $[M+H]^+$; found: 235.40.



Synthesis of *p*-chloroacetamide phenylalanine (pCaaF): N-Boc-O-*t*-Bu-4-aminophenylalanine (**1**) (0.7 g, 2.1 mmol) was dissolved in dichloromethane (7 mL). To the reaction flask was added triethylamine (0.8 mL, 6.2 mmol, 3.0 equiv), followed by the dropwise addition of chloroacetyl chloride (0.3 mL, 3.1 mmol, 1.5 equiv). The reaction was allowed to stir at RT for 3h and solvent was removed via rotary evaporation. The reaction mixture was taken up in EtOAc (20 mL) and transferred to a separatory funnel and the reaction was extracted with ddH_2O (30 mL). The aqueous

phase was extracted with 3 X 20 mL of EtOAc. Organic layers were pooled, washed with brine and dried over Na_2SO_4. Solvent was removed via rotary evaporation to yield a crude yellow solid. Crude was adsorbed onto silica gel and purified via flash chromatography (10:3 Hex:EtOAc) to afford N-Boc-O-*t*-Bu-4-chloroacetamide phenylalanine (**6**) as a yellow oil. The purified product was deprotected in a 1:1 mixture of DCM:TFA (10 mL) for 5h at RT. Volatiles were removed by rotary evaporation. The reaction mixture was taken up in Et_2O (20 mL) and removed via rotary evaporation. This process was repeated 5X to remove residual TFA. The deprotected product was taken up in 10 mL of 50mM HCl, flash frozen, and lyophilized to afford **7** as a yellow solid (0.49g, 92% yield). ^{1}H NMR (400 MHz, MeOD) δ= 7.43 (d, *J*= 8.8 Hz, 2H), 7.12 (d, *J*= 8.4 Hz, 2H), 4.08 (dd, *J*= 7.6, 5.6 Hz, 1H), 4.04 (s, 2H), 3.14 (dd, *J*= 12.4, 5.6 Hz, 1H), 3.00 (dd, *J*= 14.4, 7.6 Hz, 1H); ^{13}C NMR (126 MHz, MeOD) δ= 170.67, 166.99, 138.18, 131.30, 130.41, 121.40, 54.52, 43.37, 36.10; MS (ESI): calcd for $C_{11}H_{14}ClN_2O_3$: 257.07 $[M+H]^+$; found: 257.67.

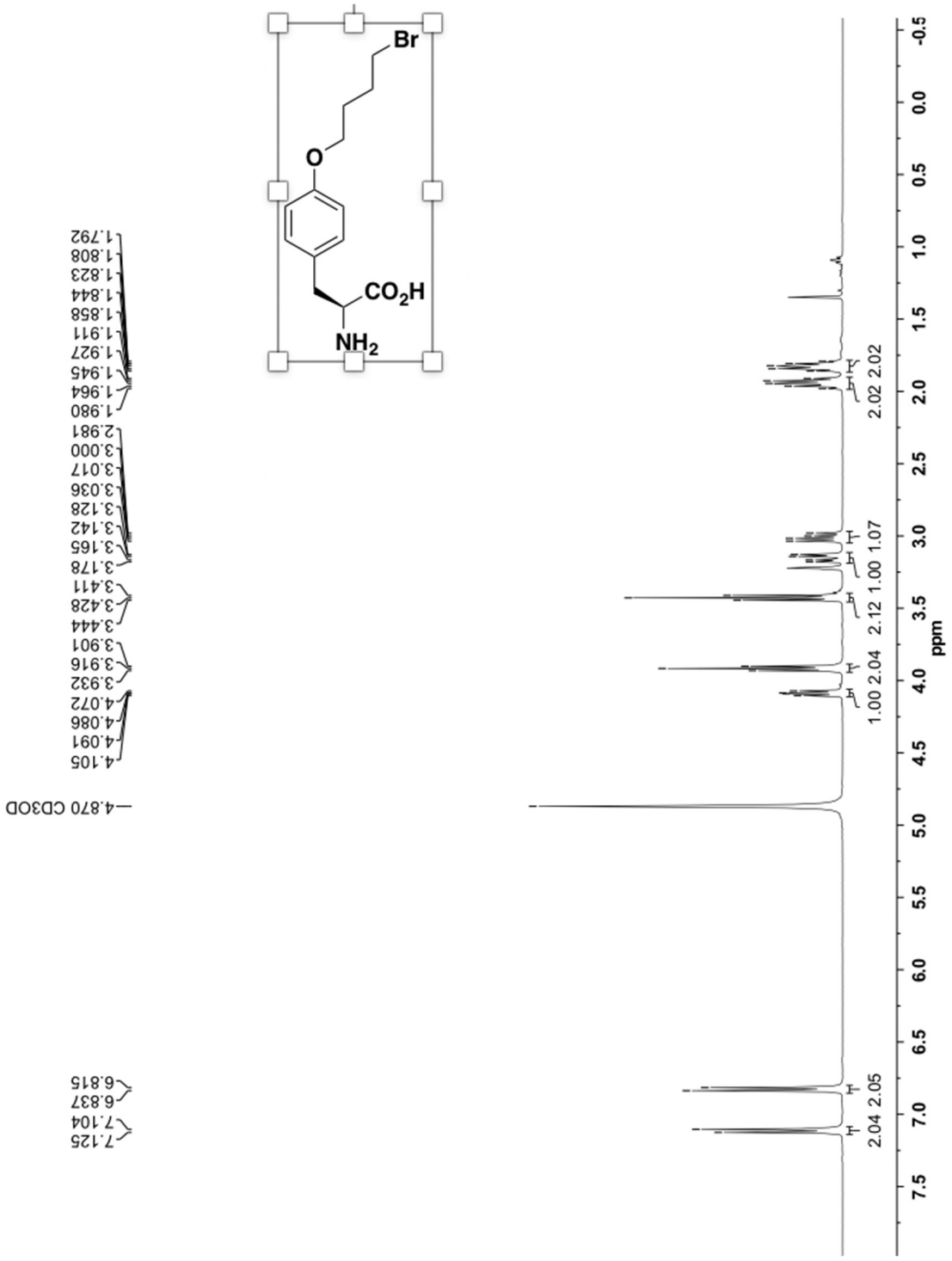


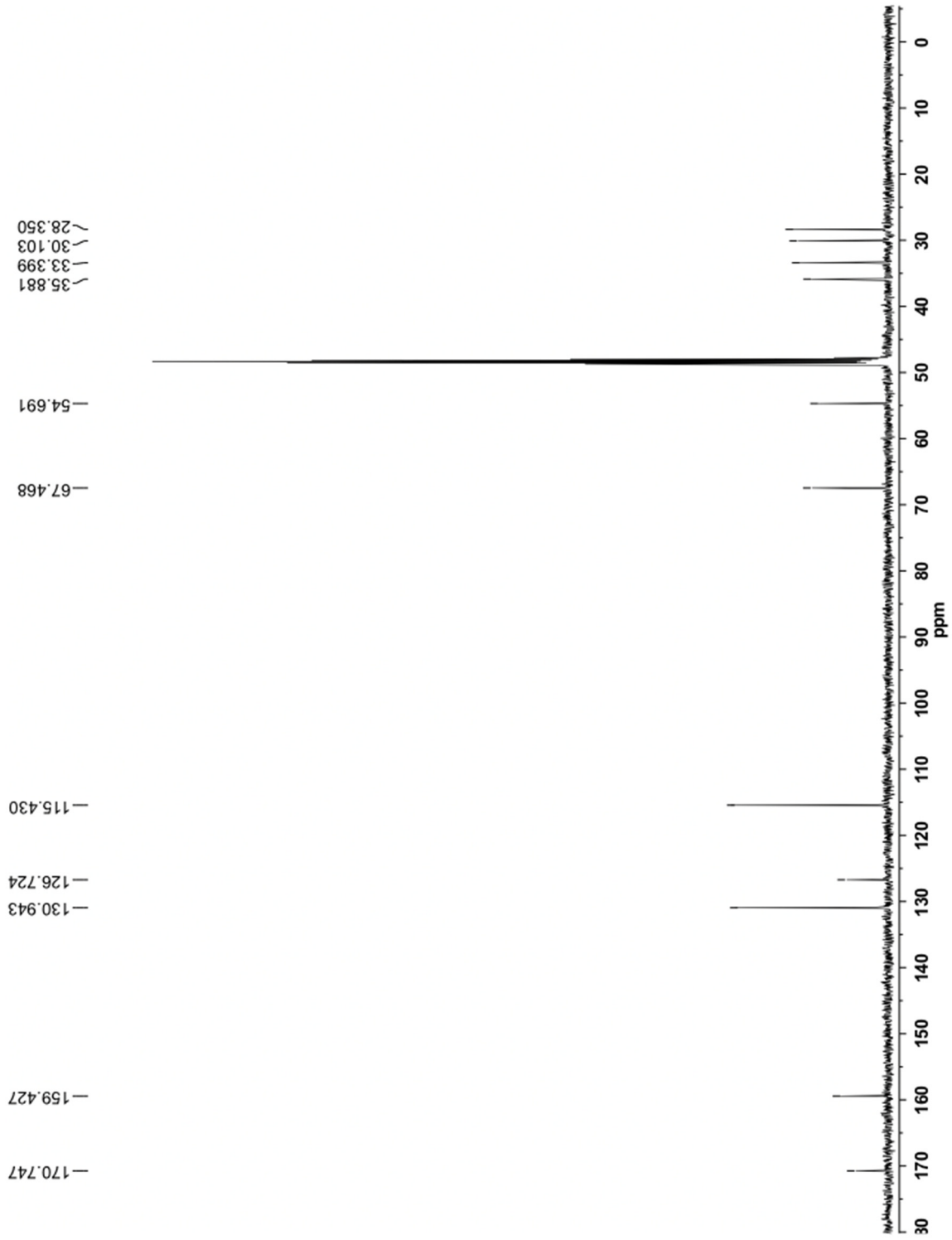
Synthesis of O-4-bromobutyl tyrosine (O4bbY): N-Boc-O-*t*-Bu-tyrosine (**8**) (1.0g, 2.9 mmol) was dissolved in EtOH (10mL). To the reaction flask was added K_2CO_3 (1.2g, 8.8 mmol, 3.0 equiv) and 1,4-dibromobutane (3.5 mL, 29.6 mmol, 10 equiv). The reaction flask was fitted with a reflux condenser, placed in an oil bath, and allowed to stir at reflux overnight and solvent was removed

via rotary evaporation. The reaction mixture was taken up in EtOAc (30 mL) and transferred to a separatory funnel and the reaction was extracted with ddH_2O (50 mL). The aqueous phase was extracted with 3 X 20 mL of EtOAc. Organic layers were pooled, washed with brine and dried over Na_2SO_4. Crude was purified via flash chromatography (10:1 Hex:EtOAc) to afford N-Boc-O-*t*-Bu-O-4-bromobutyltyrosine (**9**) as a white solid. The purified product was deprotected in a 1:1 mixture of DCM:TFA (20 mL) for 5h at RT. Volatiles were removed by rotary evaporation. The reaction mixture was taken up in Et_2O (30 mL) and removed via rotary evaporation. This process was repeated 5X to remove residual TFA. The deprotected product was taken up in 10 mL of 50mM HCl, flash frozen, and lyophilized to afford **10** as a white solid (0.8g, 89% yield). ^{1}H NMR (400 MHz, MeOD) δ= 7.11 (d, *J*= 8.4 Hz, 2H), 6.83 (d, *J*= 8.8 Hz, 2H), 4.09 (dd, *J*= 7.6, 5.6 Hz, 1H), 3.92 (t, *J* = 6.4 Hz, 2H), 3.43 (t, *J*= 6.4 Hz, 2H), 3.15 (dd, *J*= 14.4, 5.2 Hz, 1H), 3.01 (dd, *J* = 14.4, 7.6 Hz, 1H), 1.98 – 1.91 (m, 2H), 1.86 – 1.79 (m, 2H); ^{13}C NMR (126 MHz, MeOD) δ= 170.75, 159.43, 130.94, 126.72, 115.43, 67.47, 54.69, 35.88, 33.40, 30.10, 28.35; MS (ESI): calcd for $C_{13}H_{18}BrNO_3$: 317.20 $[M+H]^+$; found: 317.60.

NMR Spectra:

O4bbY

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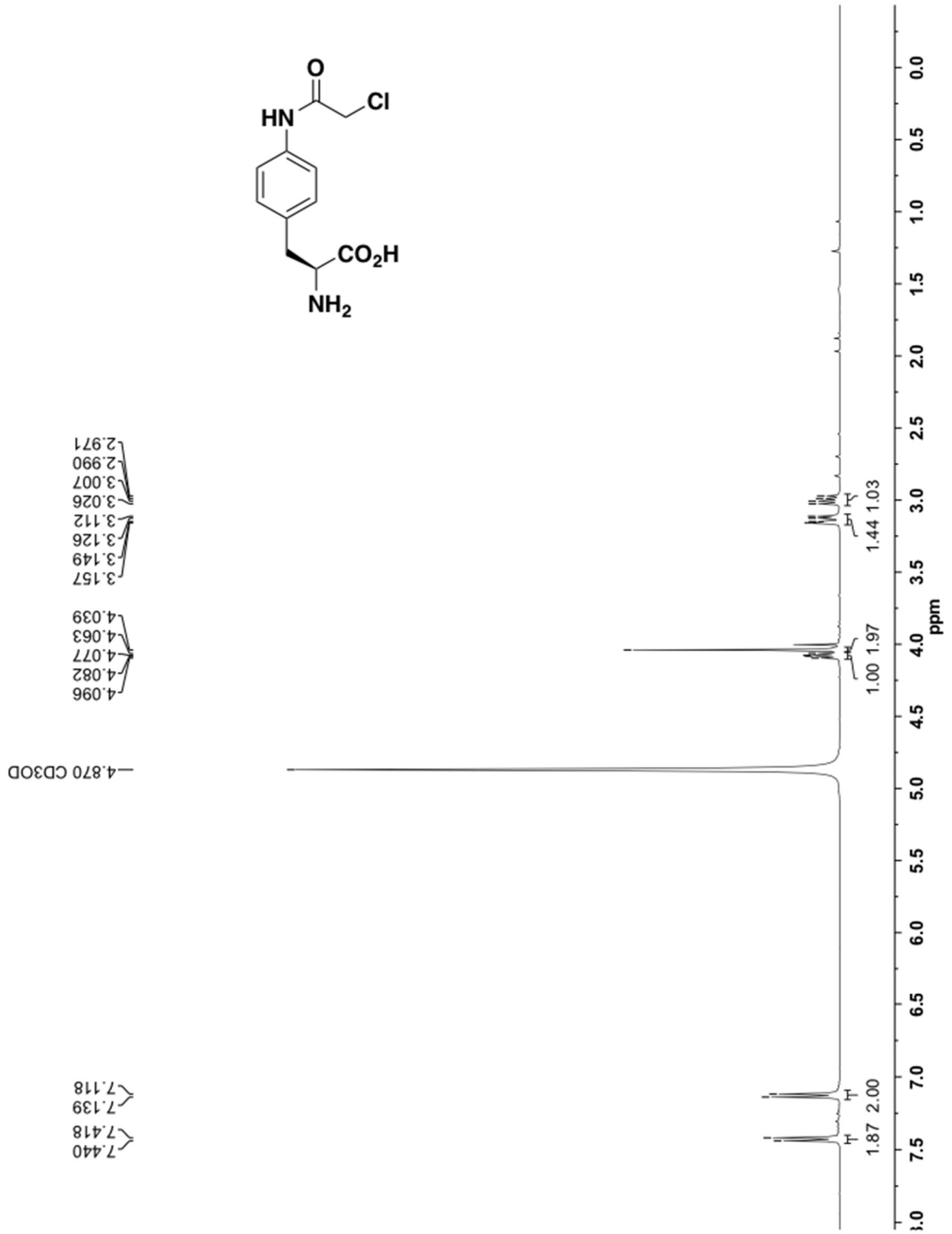
ppm

pCaaF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Cl
CO2H
NH2
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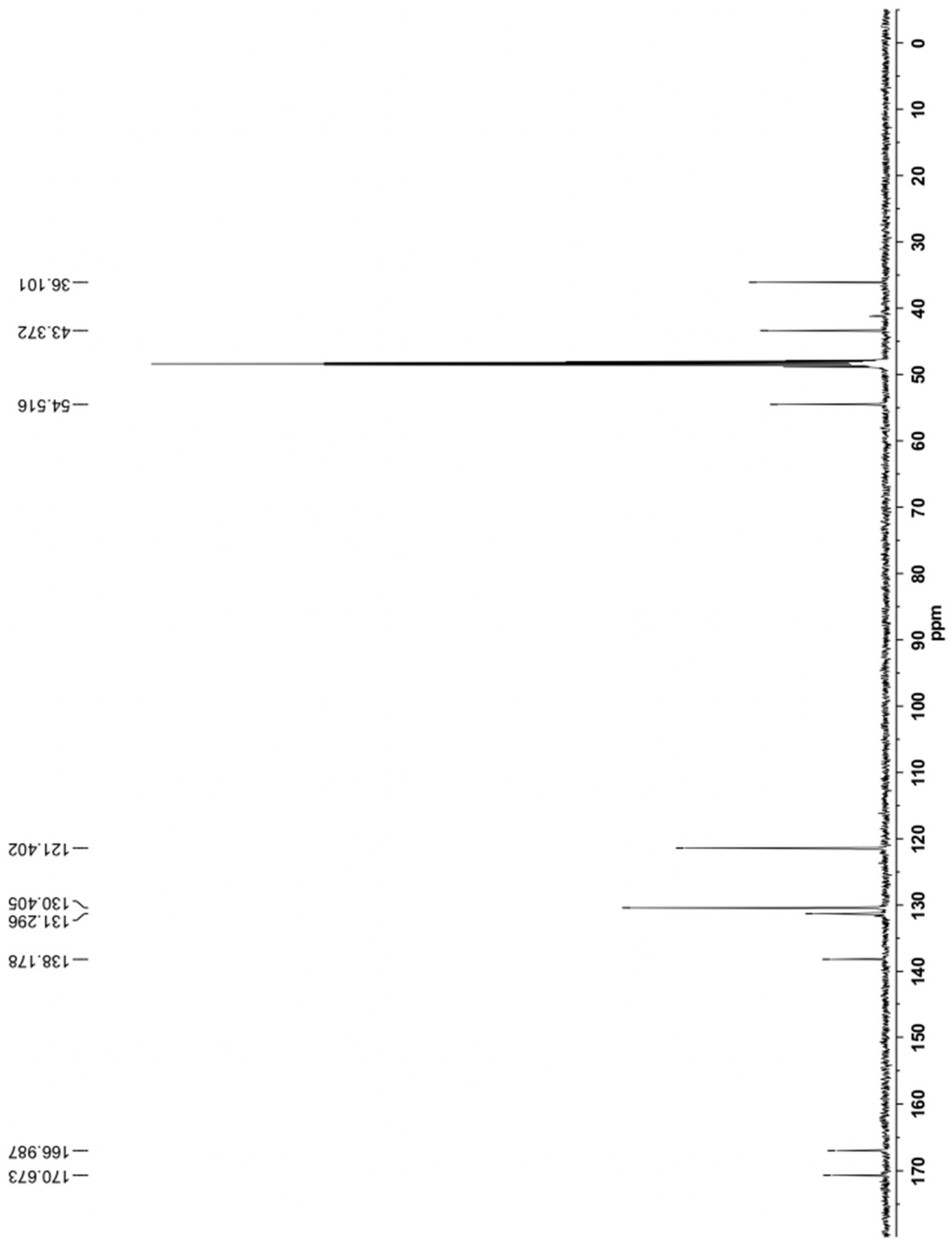
ppm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43.372
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ppm

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