Supporting Information

Cyclic peptides targeting the SARS-CoV-2 programmed ribosomal frameshifting RNA from a multiplexed phage display library

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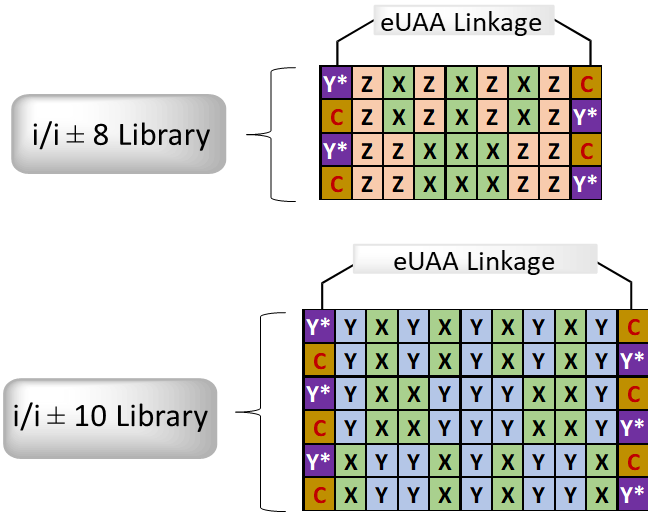
Supplemental Figures



Symbol	Codon	AAs
X	MRW	R, N, Q, H, K, S
Y	YWT	H, L, F, Y
Z	HWS	N, Q, H, I, L, K, M, F, Y

- ~6.5 X 10^7 unique DNA sequences
- ~1.4 X 10^7 unique cyclic peptides

Figure S1. Design of MOrPH-PhD Libraries. Libraries were diversified by varying the ring size, orientation of the eUAA linkage and the permutation of the degenerate codons.

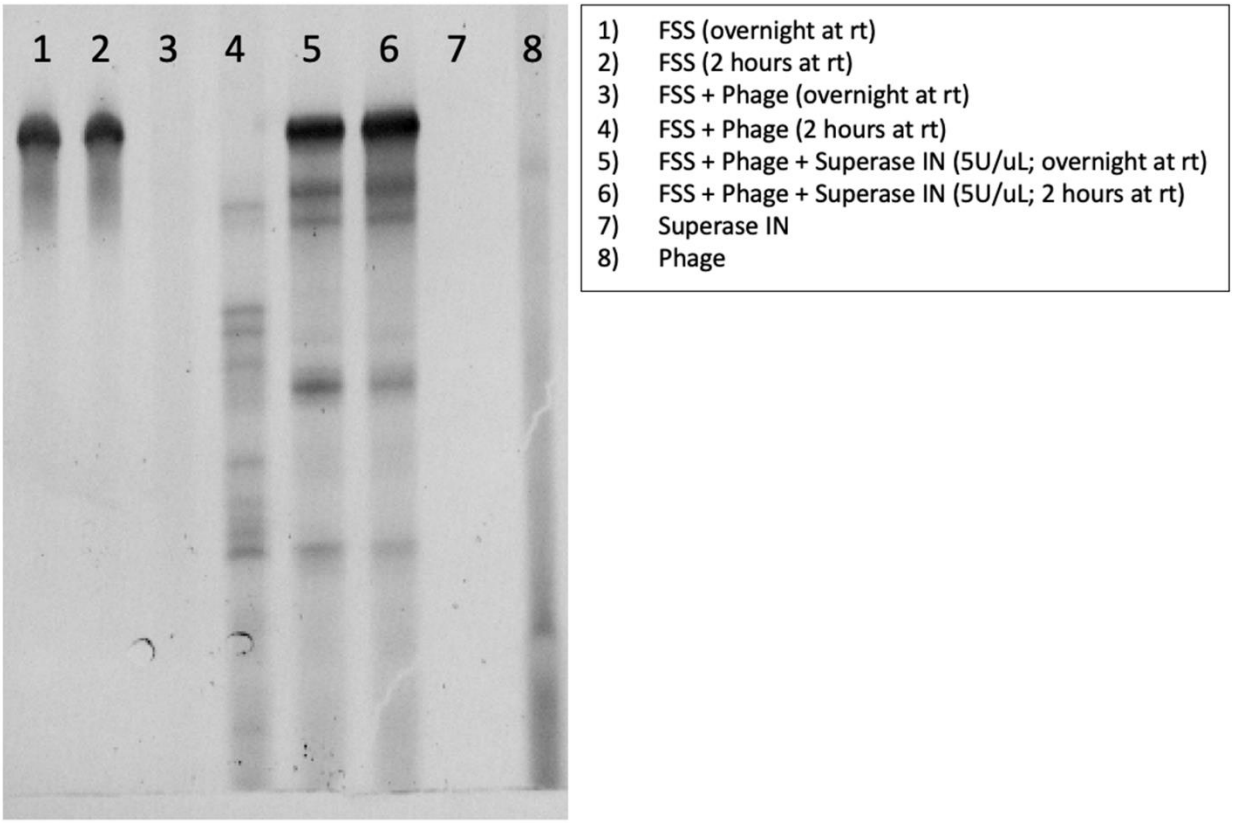


Figure S2. FSS degradation experiments. FSS PK RNA (2 mL; 250 ng/mL) (Figure 1) was incubated with 1 mL of purified phage solution used for panning experiments. Addition of a Superase IN RNase inhibitor (5 U/mL) resulted in significant reduction in FSS degradation after incubation at room temperature (rt) for 2 h (lane 6) and overnight (lane 5).

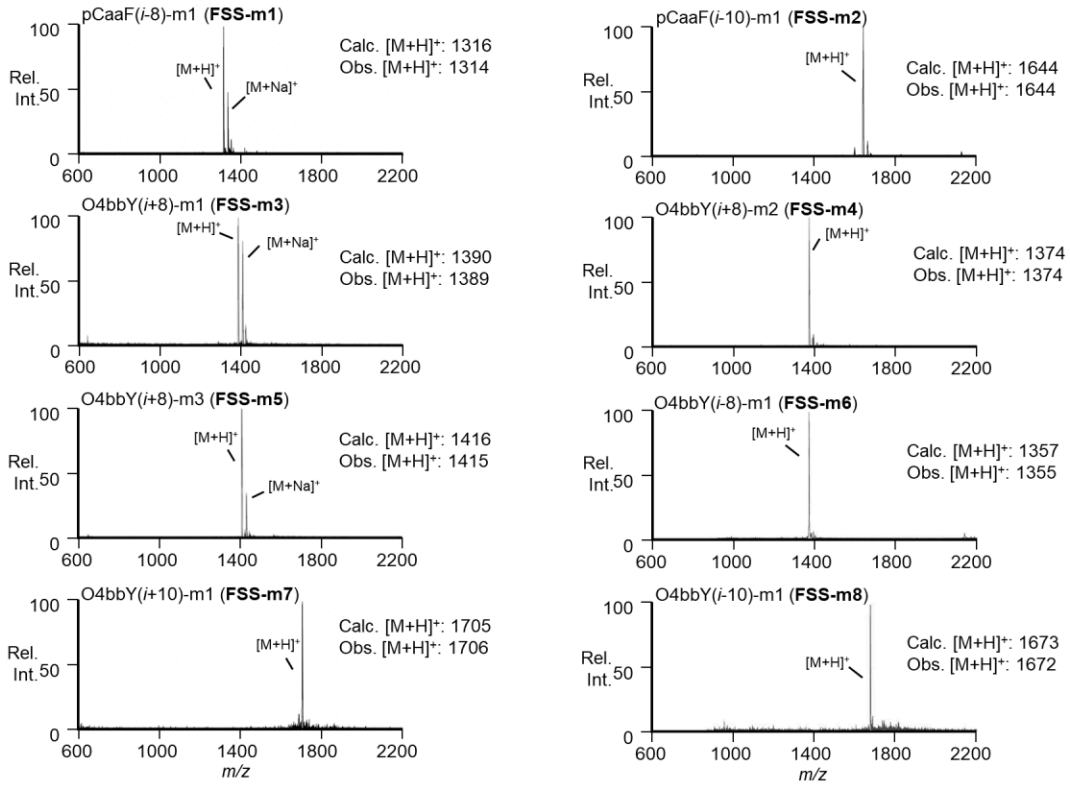


Figure S3. MALDI-TOF-MS spectra of purified synthetic cyclic peptides.

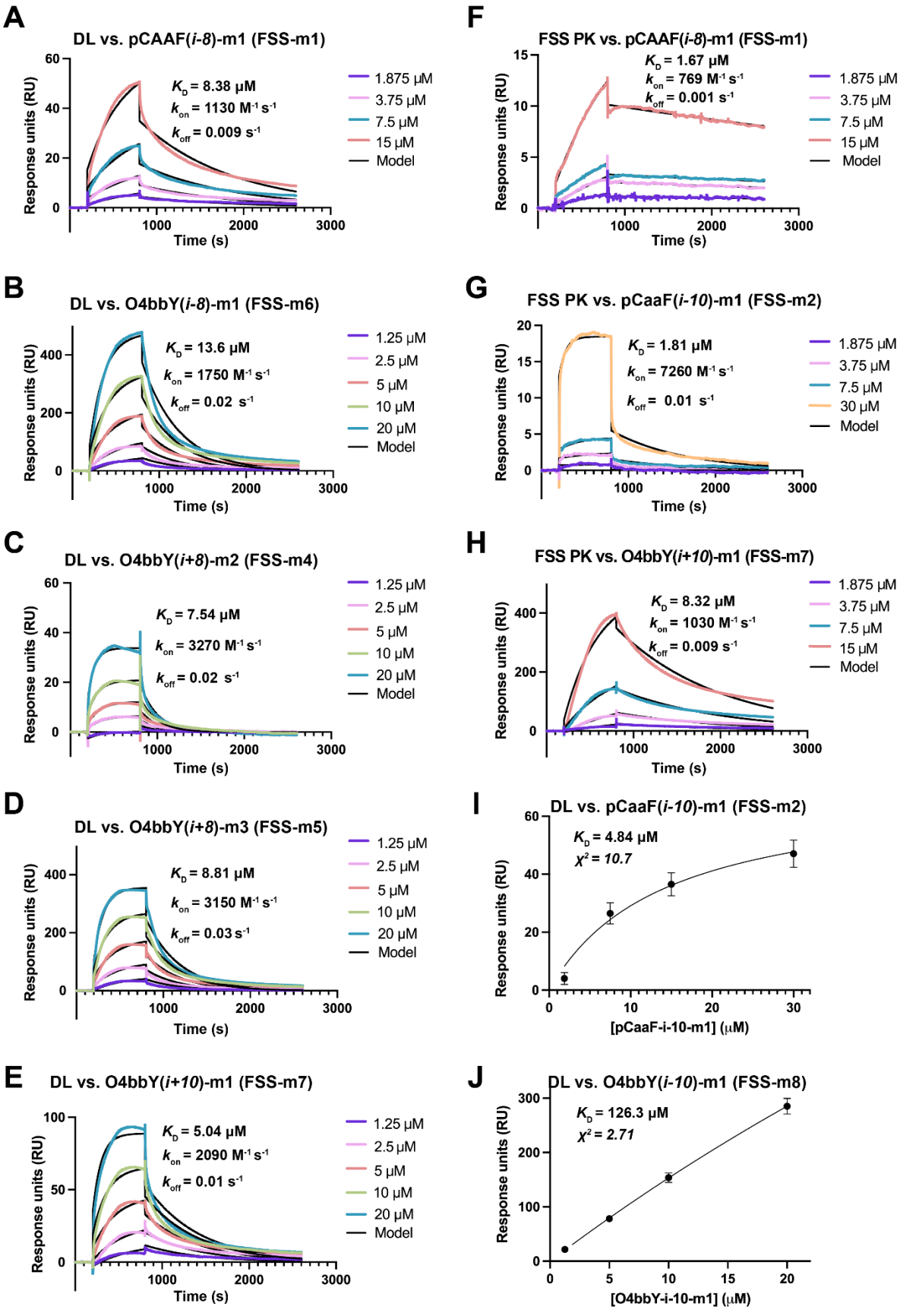

A
DL vs. pCAAF(i-8)-m1 (FSS-m1)
K_D = 8.38 μM
k_on = 1130 M^-1 s^-1
k_off = 0.009 s^-1
1.875 μM
3.75 μM
7.5 μM
15 μM
Model
Response units (RU)
Time (s)
B
DL vs. O4bbY(i-8)-m1 (FSS-m6)
K_D = 13.6 μM
k_on = 1750 M^-1 s^-1
k_off = 0.02 s^-1
1.25 μM
2.5 μM
5 μM
10 μM
20 μM
Model
C
DL vs. O4bbY(i+8)-m2 (FSS-m4)
K_D = 7.54 μM
k_on = 3270 M^-1 s^-1
k_off = 0.02 s^-1
D
DL vs. O4bbY(i+8)-m3 (FSS-m5)
K_D = 8.81 μM
k_on = 3150 M^-1 s^-1
k_off = 0.03 s^-1
E
DL vs. O4bbY(i+10)-m1 (FSS-m7)
K_D = 5.04 μM
k_on = 2090 M^-1 s^-1
k_off = 0.01 s^-1
F
FSS PK vs. pCAAF(i-8)-m1 (FSS-m1)
K_D = 1.67 μM
k_on = 769 M^-1 s^-1
k_off = 0.001 s^-1
G
FSS PK vs. pCaaF(i-10)-m1 (FSS-m2)
K_D = 1.81 μM
k_on = 7260 M^-1 s^-1
k_off = 0.01 s^-1
30 μM
H
FSS PK vs. O4bbY(i+10)-m1 (FSS-m7)
K_D = 8.32 μM
k_on = 1030 M^-1 s^-1
k_off = 0.009 s^-1
I
DL vs. pCaaF(i-10)-m1 (FSS-m2)
K_D = 4.84 μM
χ^2 = 10.7
[pCaaF-i-10-m1] (μM)
J
DL vs. O4bbY(i-10)-m1 (FSS-m8)
K_D = 126.3 μM
χ^2 = 2.71
[O4bbY-i-10-m1] (μM)

Figure S4. Representative kinetic and equilibrium binding sensorgrams and curve fits of cyclic peptide binding to the dimerization loop or full-length frameshift stimulatory sequence pseudoknot. (a) pCaaF(*i*-8)-m1 (**FSS-m1**) association with and dissociation from immobilized SARS-CoV-2 dimerization loop (DL). Here and elsewhere peptide concentrations are shown in the key; colored lines represent background subtracted data; black lines indicate the local fit to a 1:1 binding model. For this and other experiments, the average k_{on} and k_{off} rate constants and apparent K_D value from replicate runs are reported in Tables 1 and 2. (b) O4bbY(*i*+8)-m1 (**FSS-m3**) association and dissociation from immobilized DL. (c) O4bbY(*i*+8)-m2 (**FSS-m4**) association and dissociation from immobilized DL. (d) O4bbY(*i*+8)-m3 (**FSS-m5**) association and dissociation from immobilized DL. (e) O4bbY(*i*+10)-m1 (**FSS-m7**) association and dissociation from immobilized DL. (f) pCaaF(*i*-8)-m1 (**FSS-m1**) association and dissociation from immobilized SARS-CoV-2 67-mer frameshifting stimulatory sequence (FSS). (g) pCaaF(*i*-10)-m1 (**FSS-m2**) association and dissociation from immobilized SARS-CoV-2 67-mer FSS PK. (h) O4bbY(*i*+10)-m1 (**FSS-m7**) association and dissociation from immobilized SARS-CoV-2 67-mer FSS. (i) Equilibrium binding analysis of pCaaF(*i*-10)-m1 (**FSS-m2**) interacting with immobilized DL. (j) Equilibrium binding analysis of O4bbY(*i*-10)-m1 (**FSS-m8**) interacting with immobilized DL.

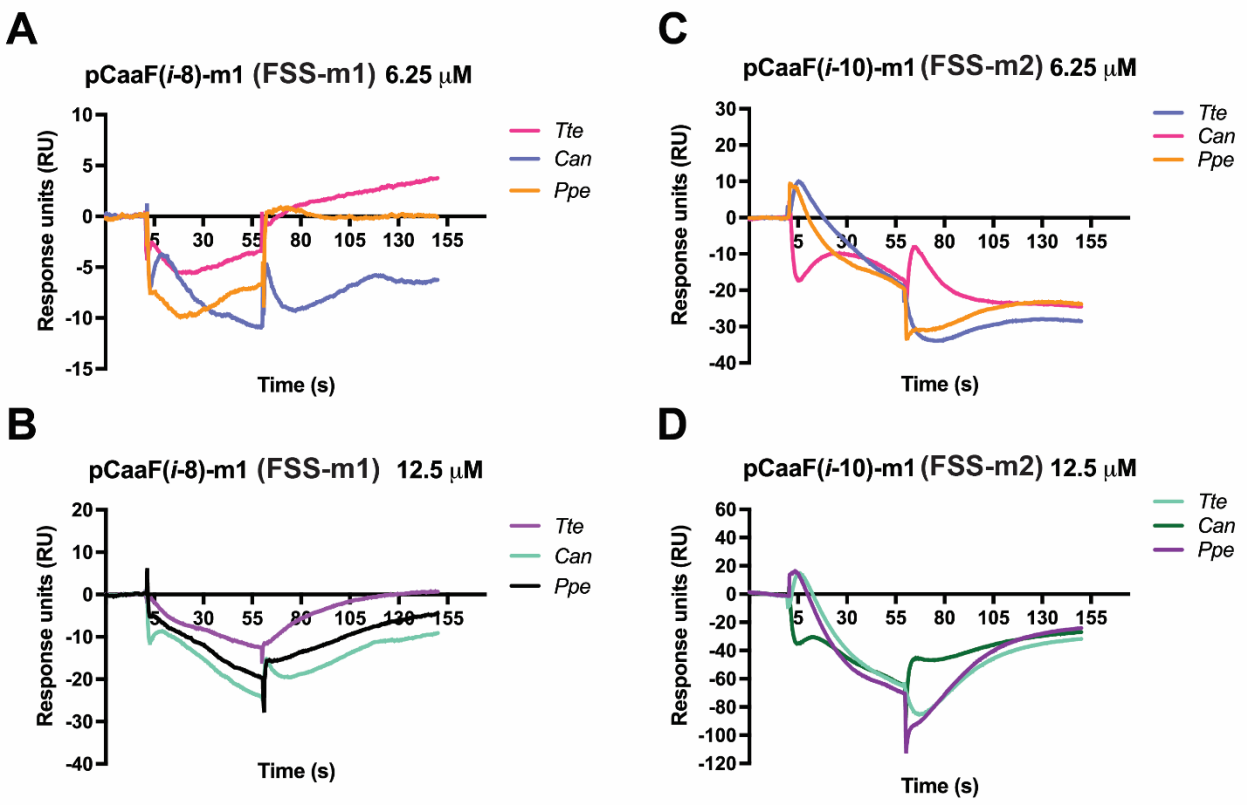


Figure S5. Representative sensorgrams for cyclic peptide binding to off-target RNAs known to adopt pseudoknot folds. (a-b) 6.25 μM or 12.5 μM pCaaF(*i*-8)-m1 (**FSS-m1**) was passed over the *Tte* (preQ$_1$-I type II), *Can* (preQ$_1$-I type I) and *Ppe* (guanine-I variant) riboswitches, which were immobilized on the chip. (c-d) 6.25 μM or 12.5 μM pCaaF(*i*-10)-m1 (**FSS-m2**) was passed over the *Tte* (preQ$_1$-I type II), *Can* (preQ$_1$-I type I) and *Ppe* (guanine-I variant) riboswitches. The absence of specific binding in the sensorgrams is apparent from the negative response units, which result from subtracting the reference cell signal from the sample cell to account for non-specific analyte interactions with the chip wherein no RNA was immobilized. In each case, there is no apparent binding as the cyclic peptide is flowed over a chip containing folded, immobilized riboswitch RNA.

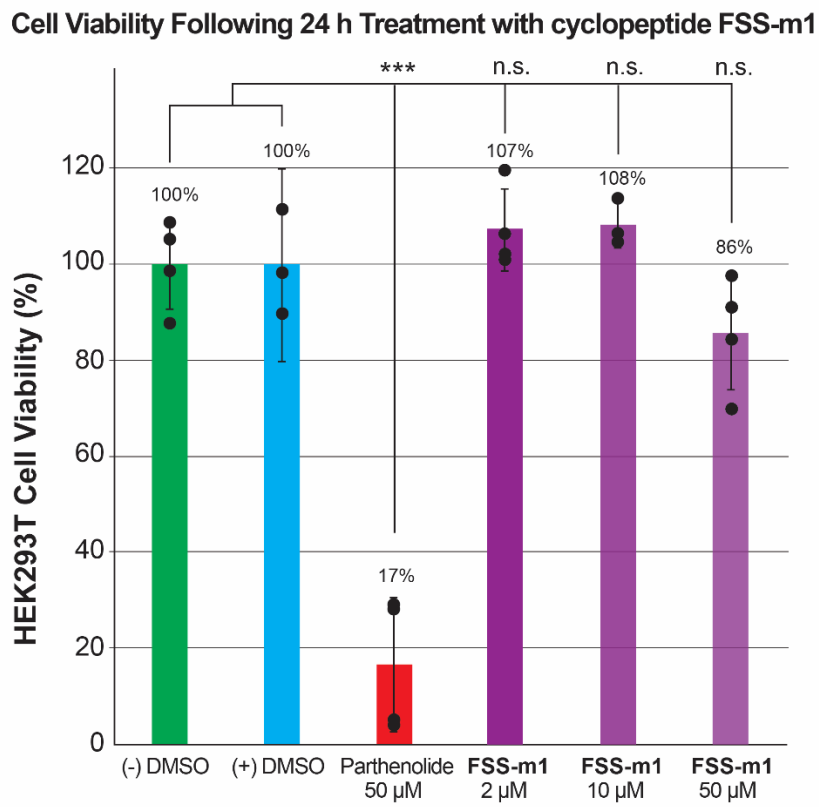
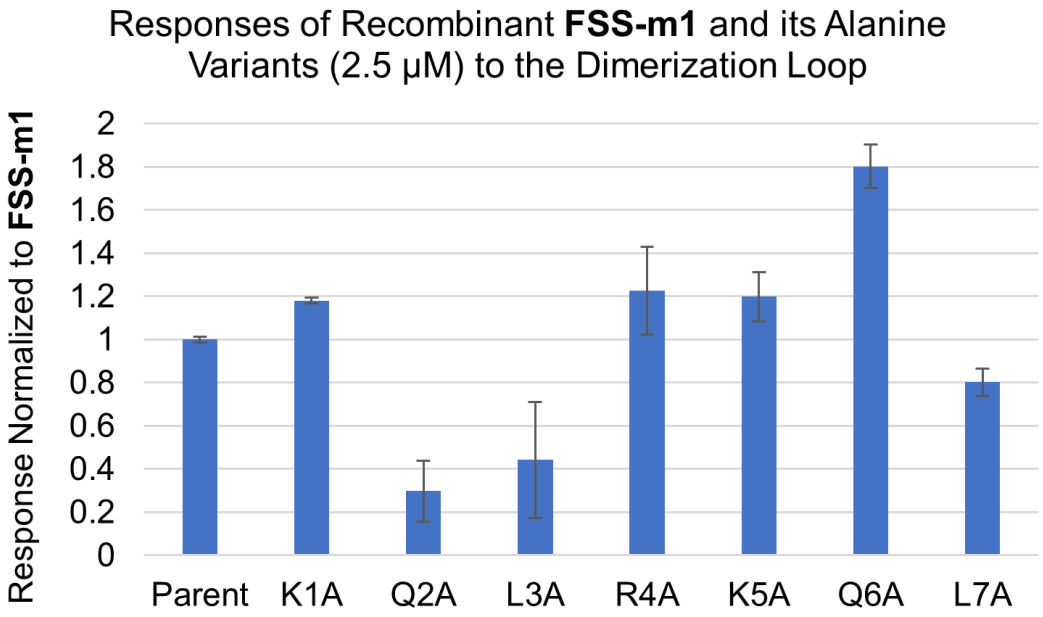
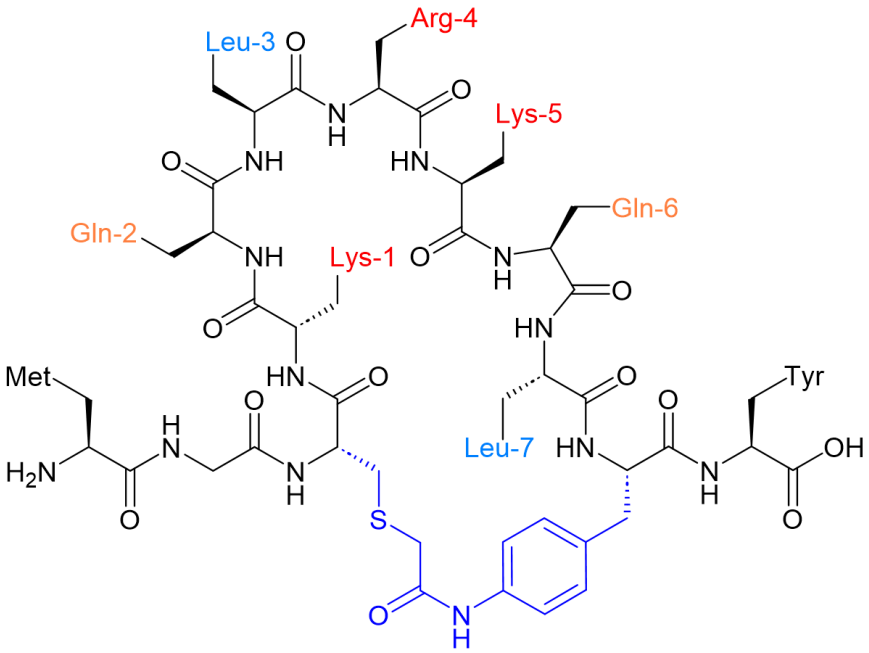


Figure S6. Viability of HEK293T cells upon treatment (24 h) with cyclopeptide **FSS-m1**. (-) DMSO = untreated cells. (+) DMSO = cells treated with vehicle only (cell culture medium +1% (v/v) DMSO). Cells were treated with parthenolide at 50 µM (positive control) or **FSS-m1** at a concentration of 2 µM, 10 µM or 50 µM in vehicle. Analyses were conducted at least in triplicate. No statistically significant difference in cell viability (n.s.) was observed for all three concentrations of **FSS-m1** compared to control cells grown in the absence (p = 0.11 for 50 µM **FSS-m1**) or presence of DMSO (p = 0.17 for 50 µM **FSS-m1**). By contrast, a statistically significant decrease in cell viability was observed for cells grown in the presence of 50 µM parthenolide ($p = 6 \times 10^{-5}$ vs. (-) DMSO cells and $p = 4 \times 10^{-4}$ vs. (+) DMSO cells).

a



b



c

	Parent	K1A	Q2A	L3A	R4A	K5A	Q6A	L7A
Avg	1	1.18	0.30	0.44	1.23	1.20	1.80	0.80
SD	0.01	0.01	0.14	0.27	0.20	0.11	0.10	0.06

Figure S7. Binding of recombinant **FSS-m1** and its alanine variants to the dimerization loop. (a) Relative response of the alanine variants of recombinant peptide **FSS-m1** (pCaaF(*i*-8)-m1) normalized to the parent peptide based on the SPR response value for each of the peptides at a concentration of 2.5 μM upon binding to the dimerization loop. Each bar represents the average of two replicates with standard deviations shown. (b) Chemical structure of the parent recombinant peptide **FSS-m1**. (c) Average and standard deviation values of the normalized SPR response for each variant.

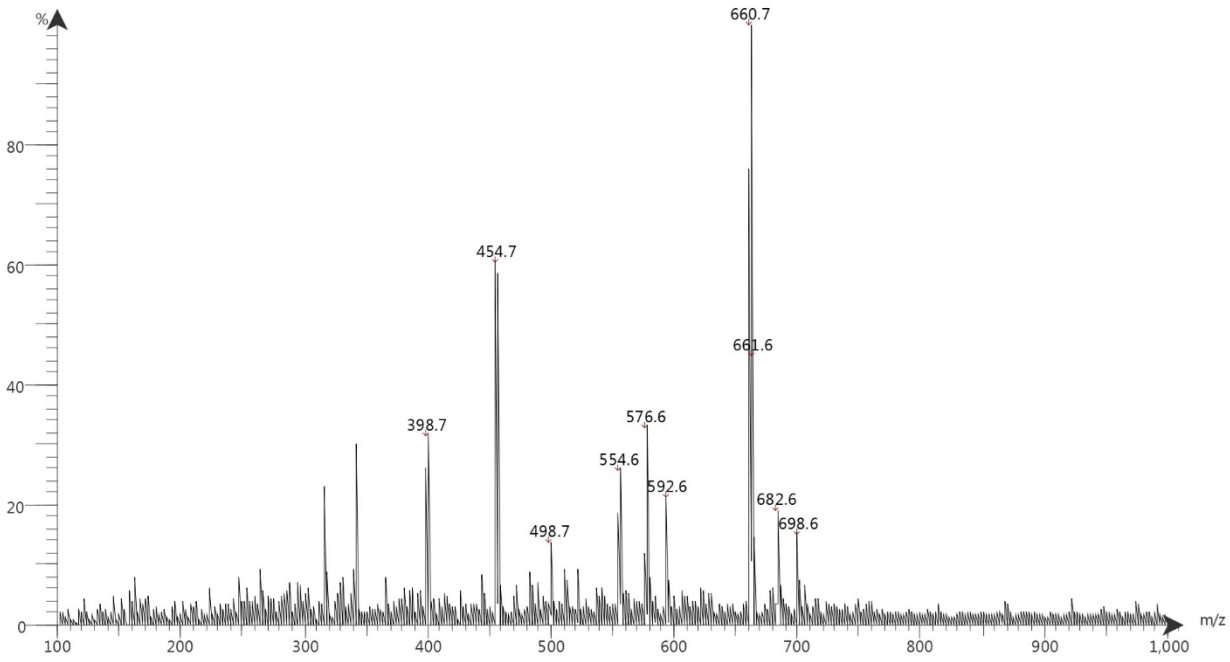
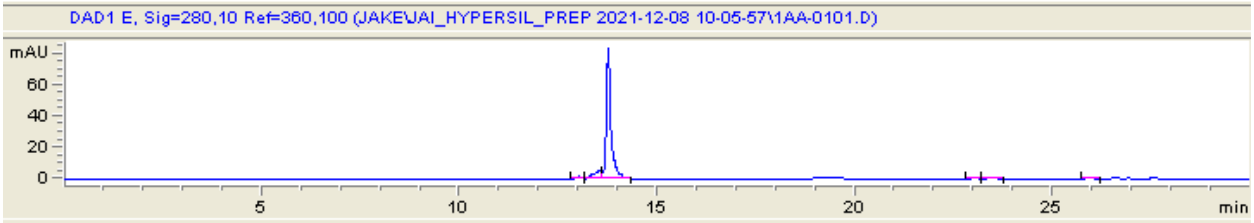


Figure S8. LC/MS spectrum for dipeptide **6**. Calc. $[M+H]^+$ *m/z* = 661.8 Da, obs. $[M+H]^+$ *m/z* = 661. 6 Da.

Figure S9. Representative high-performance liquid chromatography (HPLC) for the synthetic peptides.

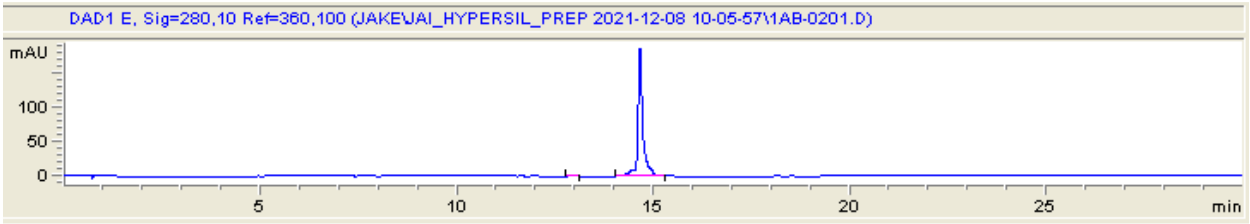
FSS-m1

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: 90%



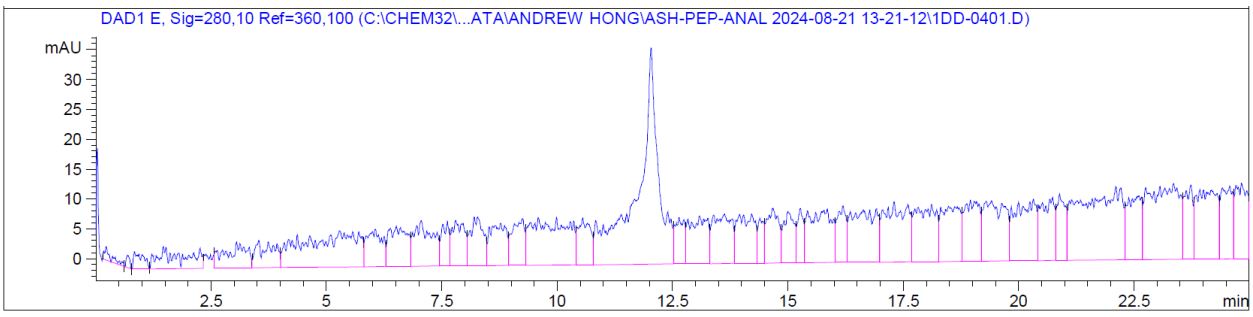
FSS-m2

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: 92%



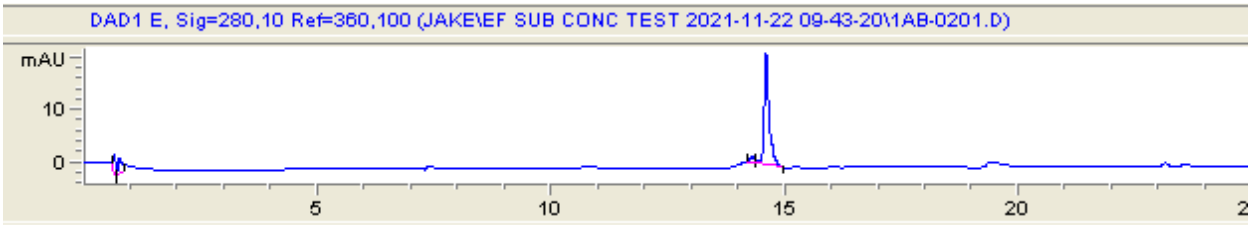
FSS-m3

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: 90%



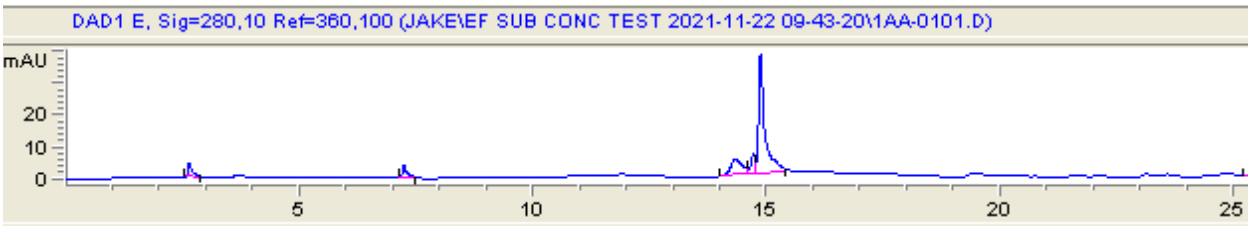
FSS-m4

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: >90%



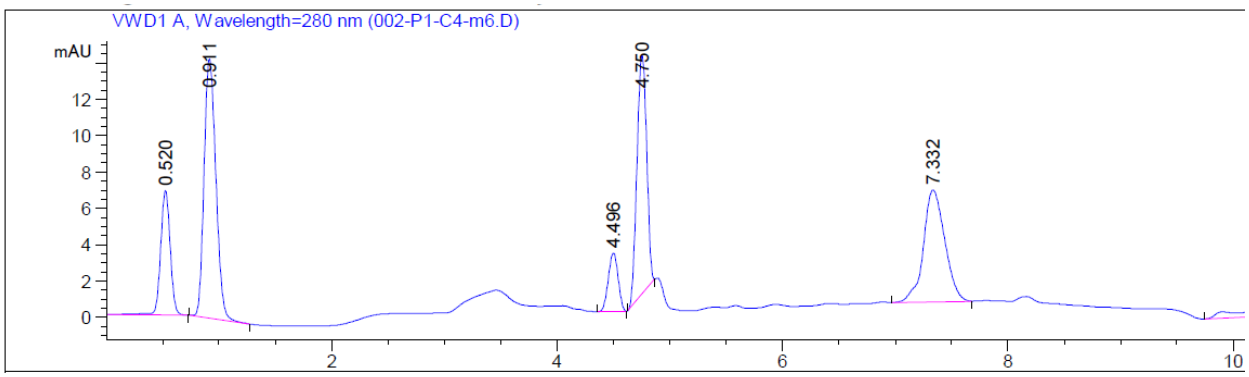
FSS-m5

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: >80%



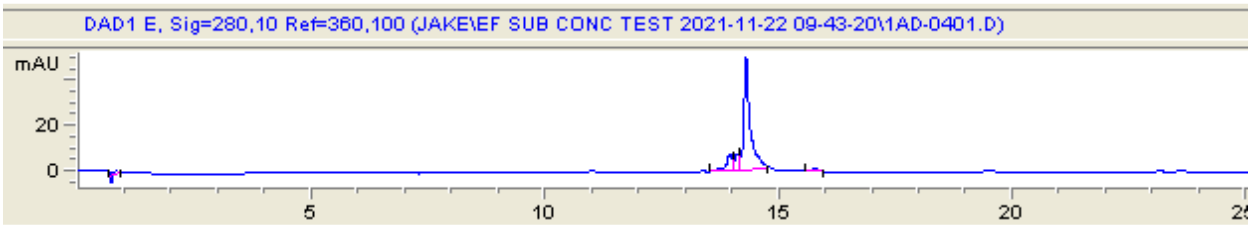
FSS-m6

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% formic acid)/ddH_2O (0.1% formic acid), *t* = 2–7 min. Purity: 80%



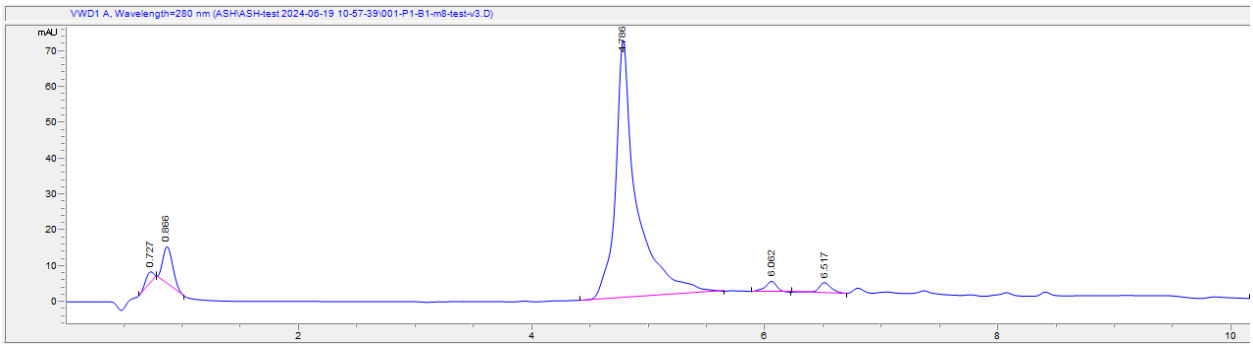
FSS-m7

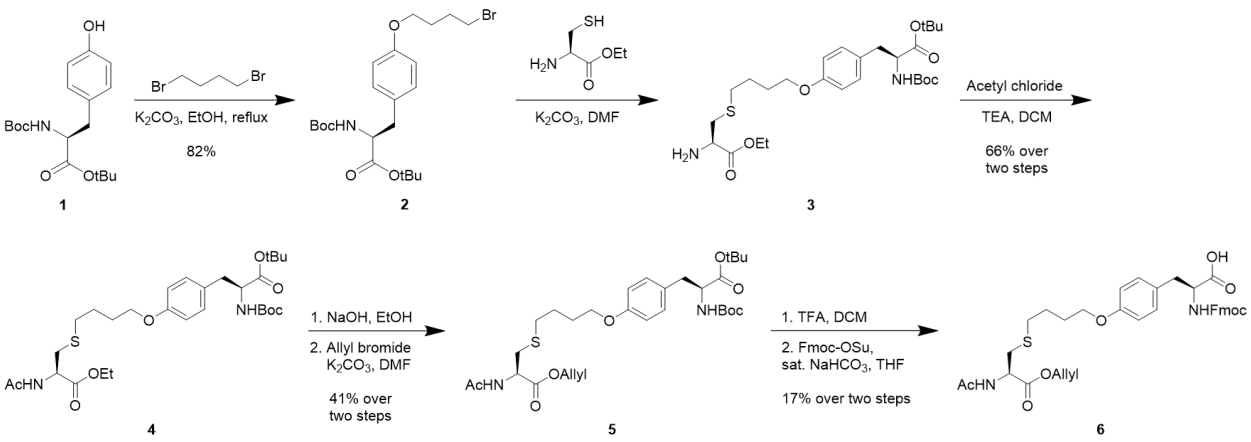
C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% TFA)/ddH_2O (0.1% TFA), *t* = 1–21 min. Purity: 80%



FSS-m8

C18 RP-HPLC method: Linear gradient of 5–95% Acetonitrile (0.1% formic acid)/ddH_2O (0.1% formic acid), *t* = 2–7 min. Purity: >95%





Scheme S1. Synthesis of dipeptide 6.

Supplemental Tables

Table S1. Oligonucleotide sequences for the preparation of MOrPH-PhD libraries.

Primer Number	Primer Name	Primer Sequence 5' → 3'
1	pSEX81-QC Reverse	caagcccaataggaacccatgtaccgtaacac
2	pSEX81-QC Forward	gaagcggaagagcgcccaatacg
3	Library1 i/i+10 Forward	ggttcttagywtmrwywtmrwywtmrwywtmrwywt tgcggttctgcggccgctggatccaaagatatc
4	Library1 i/i-10 Forward	ggttcttgcywtmrwywtmrwywtmrwywtmrwywt tagggttctgcggccgctggatccaaagatatc
5	Library2 i/i+10 Forward	ggttcttagywtmrwmrwywtywtywtmrwmrwywt tgcggttctgcggccgctggatccaaagatatc
6	Library2 i/i-10 Forward	ggttcttgcywtmrwmrwywtywtywtmrwmrwywt tagggttctgcggccgctggatccaaagatatc
7	Library3 i/i+10 Forward	ggttcttagmrwywtywtmrwywtmrwywtywtmrw tgcggttctgcggccgctggatccaaagatatc
8	Library3 i/i-10 Forward	ggttcttgcmrwywtywtmrwywtmrwywtywtmrw tagggttctgcggccgctggatccaaagatatc
9	Library4 i/i+8 Forward	ggttcttaghwsmrwhwsmrwhwsmrwhwstgc ggttctgcggccgctggatccaaagatatc
10	Library4 i/i-8 Forward	ggttcttgchwsmrwhwsmrwhwsmrwhwstag ggttctgcggccgctggatccaaagatatc
11	Library5 i/i+8 Forward	ggttcttaghwshwsmrwmrwmrwhwshwstgc ggttctgcggccgctggatccaaagatatc
12	Library5 i/i-8 Forward	ggttcttgchwshwsmrwmrwmrwhwshwstag ggttctgcggccgctggatccaaagatatc
13	Library1 i/i+10 Reverse	agaaccgcaawrwykawrwykawrwyka wrwykawrctaagaacccgccatggccggctgagctg
14	Library1 i/i-10 Reverse	agaaccctaawrwykawrwykawrwykawrwykawrg caagaacccgccatggccggctgagctg
15	Library2 i/i+10 Reverse	agaaccgcaawrwykwykawrawrawrwykwykawr ctaagaacccgccatggccggctgagctg
16	Library2 i/i-10 Reverse	agaaccctaawrwykwykawrawrawrwykwykawrg caagaacccgccatggccggctgagctg
17	Library3 i/i+10 Reverse	agaaccgcawykawrawrwykawrwykawrawrwyk ctaagaacccgccatggccggctgagctg
18	Library3 i/i-10 Reverse	agaaccctawykawrawrwykawrwykawrawrwyk gcaagaacccgccatggccggctgagctg
19	Library4 i/i+8 Reverse	agaaccgcaswdwykswdwykswdwykswdctaagaa ccc gccatggccggctgagctg

20	Library4 i/i-8 Reverse	agaaccctaswdwykswdwykswdwykswdgcaagaaccc gccatggccggctgagctg
21	Library5 i/i+8 Reverse	agaaccgcaswdswdwykwykwykswdswdctaagaaccc gccatggccggctgagctg
22	Library5 i/i-8 Reverse	agaaccctaswdswdwykwykwykswdswdgcaagaaccc gccatggccggctgagctg

Table S2. Oligonucleotide sequences for cloning macrocyclic precursor sequences.

Primer Number	Primer Name	Primer Sequence 5' → 3'
23	FLAG-CBD_R	ctcgagcagttcgagaccgttgttacc
24	pCaaF-i-8-m1_F	agtcaggatccggttcatgcaaacagctgcgtaaacagctgtaggaattgaaggccgcaaaatcg
25	T7-terminal-ext_R	gctagttattgctcagcggtggca
26	MG-FSS-m1-Y-GyrA_F	aagcacatatgggttgcaaacagctgcgtaaacagctgtagtattgcatcacgggagat
27	MG-FSS-m1_K1A_F	tatgggttgcgcgcagctgcgtaaacag
28	MG-FSS-m1_Q2A_F	tatgggttgcaaagcgctgcgtaaacag
29	MG-FSS-m1_L3A_F	tatgggttgcaaacaggcgcgtaaacag
30	MG-FSS-m1_R4A_F	gcaaacagctggcgaaacagctgtagtatt
31	MG-FSS-m1_K5A_F	ctgcgtgcccagctgtagtattgcatc
32	MG-FSS-m1_Q6A_F	gctgcgtaaagcgctgtagtattgcatc
33	MG-FSS-m1_L7A_F	gctgcgtaaacaggcgtagtattgcatc

Table S3. Amino acid sequences of the CBD and GyrA-fused macrocycles.

Macrocycle	Amino Acid Sequence
FLAG-FSS-m1-CBD-H6	MDYKDDDDKGSGSGSCKQLRKQL(pCaaF)GIEGRKIEEGKLTNPGVSAW QVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQNNGN NGLELLEHHHHHH
MG-FSS-m1-Y-GyrA-H6	MGCKQLRKQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_K1A-Y-GyrA-H6	MGCAQLRKQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_Q2A-Y-GyrA-H6	MGCKALRKQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_L3A-Y-GyrA-H6	MGCKQARKQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_R4A-Y-GyrA-H6	MGCKQLAKQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_K5A-Y-GyrA-H6	MGCKQLRAQL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPAQAIADELTDGRFYYAKVASVTDAGVQPVYS LRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_Q6A-Y-GyrA-H6	MGCKQLRKAL(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH
MG-FSS-m1_L7A-Y-GyrA-H6	MGCKQLRKQA(pCaaF)YCITGDALVALPEGESVRIADIVPGARPNSDNAI DLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYT

	VGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVY SLRVDTADHAFITNGFVSHALEHHHHHH

Table S4. Mass spectrometry analysis of recombinant macrocycles. The Cys and eUAA residues involved the thioether linkage are highlighted in bold and underlined.

Macrocycle	Amino Acid Sequence	Calc. $[M\text{-}Met+H]^+$ (Da)	Obs. $[M\text{-}Met+H]^+$ (Da)
MG-FSS-m1-Y	MG**C**KQLRKQL**(pCaaF)**Y	1439.10	1439.1
MG-FSS-m1_K1A-Y	MG**C**AQLRKQL**(pCaaF)**Y	1382.00	1382.8
MG-FSS-m1_Q2A-Y	MG**C**KALRKQL**(pCaaF)**Y	1382.04	1384.3
MG-FSS-m1_L3A-Y	MG**C**KQARKQL**(pCaaF)**Y	1397.02	1397.0
MG-FSS-m1_R4A-Y	MG**C**KQLAKQL**(pCaaF)**Y	1353.99	1356.8
MG-FSS-m1_K5A-Y	MG**C**KQLRAQL**(pCaaF)**Y	1382.00	1388.3
MG-FSS-m1_Q6A-Y	MG**C**KQLRKAL**(pCaaF)**Y	1382.04	1382.2
MG-FSS-m1_L7A-Y	MG**C**KQLRKQA**(pCaaF)**Y	1397.02	1397.7

Experimental Methods

Molecular Cloning and Plasmid Construction

The preparation of pEVOL-based plasmids for the expression of orthogonal AARS/tRNA^{CUA} pairs for amber stop codon suppression with O2beY, O4bbY, pAaF or pCaaF was reported previously.[1, 2] MOrPH-PhD libraries were inserted between the pelB leader sequence and pIII phage coat protein gene in a pSEX81 phagemid via a combination of splicing by overlap extension (SOE) and QuikChange PCR (Agilent Tech. Inc) using primers **1**-**22** in Table S1. In a first PCR reaction, inserts were amplified containing the MOrPH-PhD library either as a 5´ overhang (forward primers **3**-**12** with reverse primer **1**) or a 3´ overhang (reverse primers **13**-**22** with forward primer **2**) generating a ~1000 bp PCR product. Next, a second SOE PCR reaction was performed using primers from the first PCR reaction utilizing their respective overhangs for extension (e.g., PCR product from primers **1** and **3** with PCR product from primers **2** and **13**). Primers **1** and **2** were utilized to generate an ~2000 bp PCR product with the MOrPH-PhD library at its center. In a third PCR reaction, a QuikChange protocol was used to amplify the entire pSEX81 phagemid using the PCR product from the second reaction as the primer. The resulting PCR product was incubated with *Dpn*I (10 units) at 37 °C for 2 h to remove the template phagemid. The amplified phagemid was transformed into electrocompetent TOP10F' *E. coli* cells and selected on 20 cm x 20 cm 2× YT agar plates containing ampicillin (100 mg/L) and tetracycline (5 mg/L). A colony forming unit (c.f.u.) count exceeding by at least 3-fold the size of the respective DNA library was utilized for all the libraries. Colonies were then collected from the plates and the plasmid library was isolated using a plasmid midi-prep kit (Qiagen). The purified DNA libraries were confirmed via Sanger sequencing and pooled prior to phage generation.

Phage Expression and Purification

The pSEX81-based library was transformed into TOP10F' *E. coli* cells containing the pEVOL-based plasmid by electroporation. The cells were recovered with SOC media (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, and 20 mM glucose), and incubated with shaking at 37 °C for 1 h. Cells were then transferred to a 125 mL Erlenmeyer flask containing 20 mL 2× YT media (1.6% w/v tryptone, 1.0% w/v yeast extract, 8.6 mM NaCl) supplemented with ampicillin (100 mg/L), chloramphenicol (34 mg/L), and tetracycline (5 mg/L). Cell cultures were grown overnight (12-16 h) at 37 °C, and then cells were recovered by centrifugation (4,000 × g). The cell pellet was diluted to an OD_{600} of 0.05 in fresh 2× YT media supplemented with ampicillin (100 mg/L), chloramphenicol (34 mg/L), tetracycline (5 mg/L) and allowed to reach an OD_{600} of 0.6. A volume equal to 10% of the final phage expression culture volume was infected with Hyperphage (Progen) at an MOI of 20. Hyperphage was allowed to infect the cells for 1 h at 37 °C with shaking (200 rpm) and then pelleted by centrifugation (4,000 × g). The pellet was resuspended in 20 mL of 2× YT supplemented with ampicillin (100 mg/L), chloramphenicol (34 mg/L), tetracycline (5 mg/L), kanamycin (30 mg/L), arabinose (0.06%), and the appropriate non-canonical amino acid (2 mM). Cultures were grown for 18 h at 30 °C with shaking (200 rpm) to express the desired eUAA-containing library. After expression, cell cultures were pelleted by centrifugation (4,000 × g). The resulting supernatant was isolated and incubated at pH 8.5 for 6 h to facilitate complete cyclization of macrocyclic peptides and then concentrated using an Amicon 30 kDa spin filter to a convenient volume (250–300 μL). The concentrated supernatant was then mixed with 1:4 (v/v) 20% polyethylene glycol buffer (20% polyethylene glycol, 2.5 M NaCl) at 4 °C and incubated overnight. The precipitated phage was pelleted by centrifugation (14,000 × g) for 30 min and resuspended in 200 μL PBS (10 mM Na_2HPO_4, 1.8 mM, KH_2PO_4, 137 mM NaCl,

2.7 mM KCl, pH 7.5). The fully resuspended phage solution was centrifuged (14,000 × *g*) for an additional 5 min to remove any insoluble cellular debris. The clarified phage solution was purified a second time and then passed through a 0.22 μm filter and stored in HEPES buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 12 mM $MgCl_2$,) at 4 °C.

Determination of Phage Titer

10 μL aliquots of purified phage solutions were diluted serially in 10-fold dilutions with 2×YT media. 10 μL of each dilution is added to 90 μL of exponentially growing *E. coli* TOP10F' cells (OD_{600} = 0.4–0.6) in 1.7 mL microfuge tubes. The phage was allowed to infect *E. coli* cells for 1 h at 37 °C with shaking on a desktop thermoblock. 100 μL of phage infected *E. coli* was then spread on 2×YT agar plates containing ampicillin (100 mg/L) and tetracycline (5 mg/L) and grown overnight at 37 °C. The phage titer was determined by counting colony forming units.

RNA Folding

The biotinylated SARS-CoV-2 RNA was generated by chemical synthesis (Horizon Discovery) and deprotected and desalted prior to use. Approximately 100 μg of RNA was dissolved in 100 μL 0.05 M Na-HEPES, pH 7.0, and heated at 65 °C. After 3 min, the RNA was mixed with 100 μL of folding buffer warmed to 65 °C (0.05 M Na-HEPES, pH 7.0, 0.20 M NaCl, and 0.024 M $MgCl_2$) and incubated at 65 °C for 3 min. The RNA was slow cooled overnight to room temperature.

Selection of MOrPH-PhD Libraries

Streptavidin-coated magnetic beads (NEB) in 20 µl aliquots were added to two separate Eppendorf tubes and washed with HEPES buffer (3×) to remove residual storage buffer. One aliquot was incubated with 100 mL of a 1 mM solution of biotinylated DL (or FSS) in HEPES buffer for 2 h at room temperature for RNA immobilization. The beads were removed from solution via magnetic separation and rinsed with wash buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 12 mM $MgCl_2$, 0.05% Tween-20) to remove residual RNA. Both aliquots of beads were then incubated with 100 mL of blocking buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 12 mM $MgCl_2$, 0.5% BSA) for 2 h at room temperature, followed by rinsing with wash buffer (3 x 100 mL). Next, the phage solution was added to the aliquot lacking the immobilized RNA and incubated for 1 h at room temperature to remove library members which bind the matrix. The beads were then removed via magnetic separation to isolate the remaining phage library. The recovered phage solution was treated with SUPERaseIn RNase inhibitor (5 U/mL; Thermo Fisher) and incubated at room temperature for 15 min. The phage solution was then incubated with the immobilized RNA for 1 h at room temperature with gentle mixing. The beads were then removed from solution via magnetic separation, washed 3–5 times with wash buffer, and incubated with 100 µL of elution buffer (0.2 M glycine-HCl, pH 2.2, 1 mg/ml BSA) for 30 min at room temperature with gentle shaking. The elution solution was then neutralized with 20 µL of neutralization buffer (1 M Tris-HCl pH 9.1). 10 µl of the eluted phage solution was used to determine the titer of recovered phage. The remaining eluted phage was used to infect 2.5 mL mid-log TOP10F' cells (OD_{600} 0.4–0.6) in 2× YT for 1 h at 37 °C. This culture was then pelleted by centrifugation (4,000 × g) and resuspended in 5 mL of fresh 2× YT media supplemented with ampicillin (100 mg/L) and tetracycline (5 mg/L) and allowed to grow to saturation overnight at 37

°C. The plasmid was extracted from the overnight culture and the enriched plasmid pool was used to propagate new phage as described above. After three rounds of affinity selection and amplification, the enriched library was analyzed via next generation sequencing.

Next Generation Sequencing

Library amplicons for sequencing via an Illumina Miseq NGS platform were generated following Illumina's 16s amplicon generation protocol. In a first PCR reaction, the enriched phage libraries are amplified using primers containing Nextera-style tag sequences and locus-specific sequences for annealing to the pSEX81 phagemid. The resulting PCR product was purified via electrophoresis on a 1.2% agarose gel and the DNA band corresponding to the correct size (~200 bp) was isolated using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions and eluted in 30 μL of ddH_2O. In a second PCR reaction, the final library amplicon was generated using the product from the first PCR as the template and Nextera-style indexing primers for the introduction of index sequences to facilitate the multiplexed analysis and deconvolution of multiples libraries in a single sequencing run. The resulting PCR product was purified in a similar manner as previously described, and the library amplicon was submitted for sequencing. Data from the sequenced libraries were aligned and organized with respect to their relative sequence count for identification of enriched members and consensus analysis.

Reagents and Analysis

Chemical reagents and solvents were purchased from AAPPTec, Acros Organics, Chem-Impex and Sigma-Aldrich. Silica gel chromatography purifications were carried out by using AMD Silica Gel 60 230-400 mesh. NMR spectra were recorded on a Brüker Avance 500 MHz

spectrometer by using solvent peaks as the reference. LC/MS analyses were performed on an Advion expression® compact mass spectrometer. MALDI-TOF spectra were acquired on a Shimadzu AXIMA Confidence MALDI-TOF spectrometer using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Concentrations of peptides were determined with a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis spectrophotometer.

Synthesis of Electrophilic Unnatural Amino Acids

The eUAAs O2beY, O4bbY, pAaF and pCaaF used for incorporation into MOrPH-PhD libraries were prepared as previously described.[1, 2]

Synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(((allyloxy)carbonyl)-amino)phenyl)propanoic acid (N-Fmoc-N´-Alloc-*p*-amino-L-Phe-OH)

The SPPS building block for the preparation of Cys/pCaaF peptides was prepared as previously described.[3] R_f = 0.5 (1:9:0.1 methanol:DCM:acetic acid). ^{1}H NMR (400 MHz, MeOD) δ 9.20 (s, 1H), 7.79 (d, *J* = 7.8 Hz, 2H), 7.60 (d, *J* = 7.8 Hz, 2H), 7.38 (m, 4H), 7.31 (d, *J* = 7.2 Hz, 2H), 7.17 (d, *J* = 7.1 Hz, 2H), 6.00 (ddt, *J* = 17.9, 12.5, 6.3 Hz, 1H), 5.37 (d, *J* = 17.4 Hz, 1H), 5.23 (d, *J* = 10.5 Hz, 1H), 4.63 (d, *J* = 6.2 Hz, 2H), 4.41 (m, *J* = 5.4 Hz, 1H), 4.37 – 4.30 (m, 1H), 4.28 – 4.21 (m, 1H), 4.17 (t, *J* = 7.0 Hz, 1H), 3.17 (dd, *J* = 13.8, 5.5 Hz, 1H), 2.91 (dd, *J* = 14.5, 9.0 Hz, 1H); ^{13}C NMR (101 MHz, MeOD) δ 175.13, 158.36, 155.79, 145.18, 142.54, 134.28, 133.18, 130.70, 128.74, 128.16, 126.31, 126.20, 120.86, 119.91, 117.78, 67.94, 66.38, 56.80, 37.91. MS (ESI): calculated for $C_{28}H_{26}N_2O_6$: 487.19 $[M+H]^+$; found: 487.2.

Synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-bromobutoxy)-phenyl)propanoic acid (Fmoc-O4bbY-OH)

To an ice-cold reaction mixture of O4bbY (125 mg, 0.395 mmol, 1 equivalent) dissolved in saturated sodium bicarbonate (10 mL) at pH 9, a solution of *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide (120 mg, 0.356 mmol, 0.9 equivalent) in tetrahydrofuran (4 mL) was added dropwise. After the reaction was stirred overnight at room temperature, the reaction mixture was quenched with 5% HCl. The reaction mixture then was partitioned between ethyl acetate (30 mL) and water (30 mL) three times, and the combined organic layers were washed with brine (30 mL) and dried over anhydrous sodium sulfate. After solvent was removed by a rotary evaporator, the crude residue was washed with hexane (10 mL) three times. Subsequently, the remaining insoluble residue was taken up in 100 mM HCl (10 mL), flash frozen and lyophilized to afford the desired product as a white solid (108 mg, 51%). R_f = 0.4 (1:9:0.1 methanol:DCM:acetic acid). ^{1}H NMR (400 MHz, MeOD) δ 7.81 (d, *J* = 7.9 Hz, 2H), 7.60 (t, *J* = 6.2 Hz, 2H), 7.40 (t, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 6.7 Hz, 2H), 7.16 (d, *J* = 6.8 Hz, 2H), 6.81 (t, *J* = 7.6 Hz, 2H), 4.45 – 4.30 (m, 2H), 4.25 – 4.08 (m, 2H), 3.96 – 3.83 (m, 2H), 3.47 (t, *J* = 6.4 Hz, 2H), 3.18 (dd, *J* = 13.4, 5.0 Hz, 1H), 2.88 (dd, *J* = 13.9, 8.0 Hz, 1H), 1.99 (tt, *J* = 7.0 Hz, 2H), 1.86 (tt, *J* = 7.0 Hz, 2H); ^{13}C NMR (151 MHz, MeOD) δ 175.64, 159.21, 158.28, 145.32, 145.20, 142.55, 131.39, 128.75, 128.16, 126.44, 126.26, 120.89, 115.46, 68.01, 57.72, 57.19, 38.05, 34.07, 30.76, 29.00, 28.23. MS (ESI): calculated for $C_{28}H_{28}BrNO_5$: 538.12 $[M+H]^+$; found: 538.1.

Synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-(((*R*)-2-acetamido-3-(allyloxy)-3-oxopropyl)thio)butoxy)phenyl)propanoic acid (Cys/O4bbY Dipeptide)

t-Butyl (S)-3-(4-(4-bromobutoxy)phenyl)-2-((tert-butoxycarbonyl)amino)propanoic acid (**2**). To a solution of commercially available *N*-Boc-L-tyrosine *t*-butyl ester (**1**) (2.00 g, 5.93 mmol) and anhydrous potassium carbonate (2.46 g, 17.8 mmol, 3 equivalents) in ethanol (200 proof, 20 mL) was added 1,4-dibromobutane (7.08 mL, 59.3 mmol, 10 equivalents) dropwise and stirred at reflux overnight in accordance with previously reported procedures.[2] R_f = 0.7 (1:3 ethyl acetate:hexane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 4.96 (d, *J* = 8.2 Hz, 1H), 4.40 (d, *J* = 8.2 Hz, 1H), 3.97 (t, *J* = 6.1 Hz, 2H), 3.49 (t, *J* = 6.6 Hz, 2H), 2.98 (m, 2H), 2.11 – 2.03 (m, 2H), 1.97 – 1.89 (m, 2H), 1.42 (d, 18H). MS (ESI): calculated for $C_{22}H_{34}BrNO_5$: 494.15 $[M+Na]^+$; found: 494.2.

t-Butyl (S)-3-(4-(4-(((R)-2-amino-3-ethoxy-3-oxopropyl)thio)butoxy)phenyl)-2-((tert-butoxycarbonyl)amino)propanoic acid (**3**). A solution of **2** (1.50 g, 3.18 mmol) in dry *N*,*N*-dimethylformamide (DMF) (3 mL) was added dropwise to a solution of L-cysteine ethyl ester hydrochloride salt (884 mg, 4.76 mmol, 1.5 equivalents) with anhydrous potassium carbonate (1.76 g, 12.7 mmol, 4 equivalents) in dry DMF (5 mL) at room temperature. The mixture was stirred overnight at room temperature under argon. Following DMF removal by rotary evaporation, the mixture was diluted with ethyl acetate (30 mL) and washed with water (50 mL) twice followed by brine (30 mL). The organic layer was dried over anhydrous sodium sulfate. After solvent was removed by a rotary evaporator, crude residue of compound **3** was produced as a yellowish brown oil and used directly for the subsequent reaction. R_f = 0.3 (ethyl acetate). MS (ESI): calculated for $C_{27}H_{44}N_2O_7S$: 541.29 $[M+H]^+$; found: 540.9.

t-Butyl (S)-3-(4-(4-(((R)-2-acetamido-3-ethoxy-3-oxopropyl)thio)butoxy)phenyl)-2-((tert-butoxycarbonyl)amino)propanoic acid (**4**). Triethylamine (1330 μL, 9.53 mmol, 3 equivalents) was added to a solution of crude **3** in dichloromethane (DCM) (20 mL). After the mixture was

cooled to 0 °C, acetyl chloride (453 μL, 6.35 mmol, 2 equivalents) was added dropwise, and the reaction was stirred for three hours at room temperature under argon. Upon quenching with water, the reaction mixture was diluted with DCM (20 mL), and the reaction mixture was washed with 5% HCl (20 mL) three times, saturated sodium bicarbonate (20 mL) three times, and once with brine (20 mL). The organic layer was dried over anhydrous sodium sulfate. After solvent was removed by a rotary evaporator, the crude residue was purified by silica gel chromatography using a solvent system of ethyl acetate/hexane (3:5) to produce compound **4** as a yellowish brown oil (1.22 g, 66% over two steps). R_f = 0.5 (3:1 ethyl acetate:hexane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.5 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 6.32 (d, *J* = 7.6 Hz, 1H), 4.97 (d, *J* = 8.7 Hz, 1H), 4.81 (m, 1H), 4.40 (m, 1H), 4.23 (m, 2H), 3.93 (t, *J* = 6.3 Hz, 2H), 3.07 – 2.91 (m, 4H), 2.59 (td, *J* = 7.2, 1.6 Hz, 2H), 2.05 (s, 3H), 1.88 – 1.82 (m, 2H), 1.78 – 1.73 (m, 2H), 1.42 (d, 18H), 1.30 (t, *J* = 7.2 Hz, 3H). MS (ESI): calculated for $C_{29}H_{46}N_2O_8S$: 605.29 $[M+Na]^+$; found: 605.0.

Allyl N-acetyl-S-(4-(4-((S)-3-(tert-butoxy)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)-phenoxy)butyl)-L-cysteine (**5**). A 2 N solution of sodium hydroxide (0.8 g) in water (10 mL) was added to a solution of **4** (1.22 g, 2.10 mmol) in ethanol (10 mL). After the reaction was stirred for 2 h at room temperature, the reaction mixture was quenched with 5% HCl. The mixture was partitioned between ethyl acetate (30 mL) and water (30 mL) three times, and the combined organic layers were washed with brine (30 mL) and dried over anhydrous sodium sulfate. Upon solvent removal via rotary evaporation, crude residue was used directly for the subsequent reaction. MS (ESI): calculated for $C_{27}H_{42}N_2O_8S$: 577.26 $[M+Na]^+$; found: 576.9. To a reaction mixture of the crude residue with anhydrous potassium carbonate (1.16 g, 8.38 mmol, 4 equivalents) in dry DMF (5 mL), allyl bromide (272 μL, 3.14 mmol, 1.5 equivalents) was added dropwise, and the reaction was stirred overnight at room temperature under argon. Upon DMF

removal via rotary evaporation, the reaction mixture was diluted with ethyl acetate (30 mL) and washed with water (30 mL) twice followed by brine (30 mL). The organic layer was dried over anhydrous sodium sulfate. After solvent was removed by rotary evaporation, the crude residue was purified by silica gel chromatography using a solvent system of ethyl acetate/hexane (2:1) to produce compound **5** as an oil (512 mg, 41% over two steps). R_f = 0.63 (3:1 ethyl acetate:hexane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 6.41 (d, *J* = 7.6 Hz, 1H), 5.96 – 5.86 (m, 1H), 5.35 (dq, *J* = 17.1, 1.4 Hz, 1H), 5.27 (dq, *J* = 10.5, 1.2 Hz, 1H), 4.99 (d, *J* = 8.4 Hz, 1H), 4.85 (dt, *J* = 7.8, 5.2 Hz, 1H), 4.66 (dt, *J* = 5.8, 1.4 Hz, 2H), 4.40 (q, *J* = 6.1 Hz, 1H), 3.93 (t, *J* = 6.2 Hz, 2H), 3.08 – 2.95 (m, 4H), 2.59 (td, *J* = 7.2, 1.4 Hz, 2H), 2.05 (s, 3H), 1.89 – 1.81 (m, 2H), 1.75 (m, 2H), 1.42 (d, 18H). MS (ESI): calculated for $C_{30}H_{46}N_2O_8S$: 617.29 $[M+Na]^+$; found: 616.9.

(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-(((R)-2-acetamido-3-(allyloxy)-3-oxopropyl)thio)butoxy)phenyl)propanoic acid (**6**). Compound **5** (512 mg, 0.861 mmol) was dissolved in a 1:1 mixture of trifluoroacetic acid (TFA) and DCM (10 mL total). After the reaction was stirred for 5 h, volatiles were removed via rotary evaporation. The sample was then suspended in diethyl ether (20 mL), and volatiles were removed via rotary evaporation. This process was performed five times to remove residual TFA, yielding crude residue that was used directly for the subsequent reaction. MS (ESI): calculated for $C_{21}H_{30}N_2O_6S$: 439.19 $[M+H]^+$; found: 438.8. To an ice-cold reaction mixture of the crude residue dissolved in saturated sodium bicarbonate (10 mL) at pH 9, a solution of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (446 mg, 1.32 mmol, 1.53 equivalents) in tetrahydrofuran (2 mL) was added dropwise. After the reaction was stirred overnight at room temperature, the reaction mixture was quenched with 5% HCl. The reaction mixture then was partitioned between ethyl acetate (30 mL) and water (30 mL)

three times, and the combined organic layers were washed with brine (30 mL) and dried over anhydrous sodium sulfate. After solvent was removed by a rotary evaporator, the crude residue was purified over silica gel chromatography using a solvent system of methanol/DCM/acetic acid (1:9:0.1) to produce compound **6** as a clear oil (95 mg, 17% over two steps). R_f = 0.19 (1:9:0.1 methanol:DCM:acetic acid). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.6 Hz, 2H), 7.56 (t, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.30 (m, 2H), 7.04 (d, *J* = 8.1 Hz, 2H), 6.78 (d, *J* = 8.3 Hz, 2H), 6.51 (t, *J* = 6.6 Hz, 1H), 5.90 (m, 1H), 5.39 – 5.31 (m, 2H), 5.26 (dd, *J* = 10.4, 1.3 Hz, 1H), 4.83 (m, 1H), 4.65 (m, 3H), 4.44 (dd, *J* = 10.5, 7.1 Hz, 1H), 4.34 (dd, *J* = 10.7, 6.9 Hz, 1H), 4.20 (t, *J* = 7.2 Hz, 1H), 3.91 (t, *J* = 6.1 Hz, 2H), 3.10 (qd, *J* = 14.0, 5.6 Hz, 2H), 2.99 (qd, *J* = 13.8, 5.1 Hz, 2H), 2.56 (m, 2H), 2.04 (m, 3H), 1.81 (m, 2H), 1.72 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.61, 170.92, 170.68, 158.13, 155.89, 143.95, 143.86, 141.45, 131.36, 130.60, 127.87, 127.20, 125.20, 120.12, 119.42, 114.82, 67.30, 67.15, 66.61, 54.86, 52.25, 47.30, 37.16, 34.16, 32.54, 28.25, 26.19, 23.08. MS (ESI): calculated for $C_{36}H_{40}N_2O_8S$: 661.26 $[M+H]^+$; found: 660.7 (Figure S6; Scheme S1).

Synthesis of pCaaF-*i*/*i*-(8/10) Macrocyclic Peptides via On-Resin Cyclization

Synthesis was carried out by conventional Fmoc-solid-phase peptide synthesis on Knorr amide resin (0.4 mmol g^{-1} on a 0.1-mmol scale). Peptides were generated manually in syringes equipped with a Teflon frit. Initially, the resin was subjected to swelling for 30 min in 5 ml of a 1:1 mixture of dichloromethane/*N*,*N*-dimethylformamide (DCM/DMF). Swollen resin was then treated with 20% piperidine in DMF containing 0.05 M 1-hydroxybenzotriazole (3 cycles of 3 min, 5 min, and 3 min) to achieve deprotection of the Fmoc group. After washing with DMF (5×), the resin was loaded with four equiv. of Fmoc-Gly-OH, four equiv. of 2-(6-chloro-1H-benzotriazole-

1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and eight equiv. of *N*,*N*-diisopropylethylamine (DIPEA) in DMF with respect to the initial loading of the resin. The coupling reaction was allowed to proceed at room temperature for 40 min, followed by washing with DMF (5x). Fmoc deprotection was performed as previously described and, after washing with DMF (5×), the resin was loaded with 0.5 equiv. of N-Fmoc-N´-Alloc-*p*-amino-L-Phe-OH, 0.5 equiv. of HCTU, and 1.0 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 40 min, followed by washing with DMF (5×). The remaining deprotected N-terminal amino groups were acetylated using 5 equiv. of acetic anhydride and 10 equiv. of DIPEA for 30 min followed by washing with DMF (5×). This procedure reduces the overall loading capacity of the resin by half but facilitates on-resin peptide cyclization at the final step of synthesis. Next, Fmoc groups were deprotected and the resin was washed with DMF (5×). The resin was loaded with 2 equiv. of amino acid, 2 equiv. of HCTU, and 4 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 40 min. The resin was then washed with DMF (5×) and this process was repeated to attain the desired peptide sequence. Upon coupling of Fmoc-L-Cys(Acm)-OH as the final amino acid residue in the peptide chain, the resin was treated with 0.25 equiv. of $Pd(PPh_3)_4$ and 20 equiv. of $PhSiH_3$ in DCM for 1 h at room temperature with shaking to promote deprotection of the Alloc protecting group of *p*-amino-L-phenylalanine. The resin was washed with DCM (5×) and treated with 2 equiv. of chloroacetyl chloride in DCM for 1 h to acetylate *p*-amino-L-phenylalanine and washed with DCM (5×) and DMF (2×). Next, the resin was treated with 10 equiv. of $PdCl_2$ in DMF/H_2O (9:1) and incubated overnight at room temperature with shaking to promote Acm deprotection of cysteine. The resin was then washed with DMF (5×), H_2O (2×), DMF (2×), DCM (2×), DMF (2×), and treated with 0.5 M dithiothreitol (DTT) in DMF (2× 1 min) to facilitate removal of the palladium catalyst. The resin was taken up in 5 mL of a 10%

solution of DIPEA in DMF and incubated at room temperature overnight with shaking to promote cyclization between the electrophilic unnatural amino acid (pCaaF) and cysteine. Upon final Fmoc deprotection, N-terminal amino groups were acetylated using 5 equiv. of acetic anhydride and 10 equiv. of DIPEA for 30 min followed by washing with DMF (5×). The resulting macrocyclic peptide was cleaved from the resin as follows: the resin was washed with DMF (3×), DCM (3×) and dried *in vacuo*. The dried resin was taken up in 10 mL of cleavage solution comprising 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropylsilane (TIPS) and 2.5% (v/v) H_2O and allowed to incubate for 2 h at room temperature. The filtrate was collected and concentrated under reduced pressure and mixed with a tenfold excess volume of cold diethyl ether followed by centrifugation (4,000 ×g). Supernatant was removed, and the crude peptide precipitate was dissolved in 10 mL of a 1:1 mixture of ACN/H_2O and lyophilized. The cyclic peptide was then purified via semipreparative HPLC using a linear gradient of 5% to 60% acetonitrile in water (with 0.1% TFA) over 30 min. The correct peptide mass was confirmed via matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS analysis using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix (Figure S3). Subsequently, pure fractions were combined and lyophilized. Finally, for removal of TFA, peptides were resuspended in 100 mM HCl and lyophilized to yield the peptide hydrochloride salts.

Synthesis of O4bbY-*i*/*i*+(8/10) Macrocyclic Peptides

Synthesis was carried out by conventional Fmoc-solid-phase peptide synthesis on Knorr amide resin (0.4 mmol g^{-1} on a 0.1-mmol scale). Peptides were generated manually in syringes equipped with a Teflon frit. Initially, the resin was subjected to swelling for 30 min in 5 ml of a 1:1 mixture of dichloromethane/*N*,*N*-dimethylformamide (DCM/DMF). Swollen resin was then

treated with 20% piperidine in DMF containing 0.05 M 1-hydroxybenzotriazole (3 cycles of 3 min, 5 min, and 3 min) to achieve deprotection of the Fmoc group. After washing with DMF (5×), the resin was loaded with four equivalents (equiv.) of Fmoc-Gly-OH, four equiv. of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and eight equiv. of *N*,*N*-diisopropylethylamine (DIPEA) in DMF with respect to the initial loading of the resin. The coupling reaction was allowed to proceed at room temperature for 40 min, followed by washing with DMF (5×). The Fmoc deprotection was performed as previously described and, after washing with DMF (5×), the resin was loaded with 0.5 equiv. of Fmoc-L-Cys(Acm)-OH, 0.5 equiv. of HCTU, and 1 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 40 min, followed by washing with DMF (5×). The remaining deprotected N-terminal amino groups were acetylated using 5 equiv. of acetic anhydride and 10 equiv. of DIPEA for 30 min followed by washing with DMF (5×). This procedure reduces the overall loading capacity of the resin by half and facilitates on-resin peptide cyclization at the final step of synthesis. Next, Fmoc groups were deprotected and the resin was washed with DMF (5×). The resin was loaded with 2 equiv. of amino acid, 2 equiv. of HCTU, and 4 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 40 min. The resin was then washed with DMF (5×) and this process was repeated to attain the desired peptide sequence. Upon coupling of Fmoc-O4bbY-OH as the final amino acid residue in the peptide chain, the resin was treated with 10 equiv. of $PdCl_2$ in DMF/H_2O (9:1) and incubated overnight at room temperature with shaking to promote Acm deprotection of cysteine. The resin was then washed with DMF (5×), H_2O (2×), DMF (2×), DCM (2×), DMF (2×), and treated with a 0.5 M solution of DTT in DMF (2× 1 min) to facilitate removal of the palladium catalyst. Next, the resin was taken up in 5 mL of a 10% solution of DIPEA in DMF and incubated at room temperature overnight with shaking to promote cyclization between the electrophilic

unnatural amino acid (O4bbY) and cysteine. Upon final Fmoc deprotection, N-terminal amino groups were acetylated using 5 equiv. of acetic anhydride and 10 equiv. of DIPEA for 30 min followed by washing with DMF (5×). The resulting macrocyclic peptide was cleaved from the resin as follows: the resin was washed with DMF (3×), DCM (3×) and dried *in vacuo*. The dried resin was taken up in 10 mL of cleavage solution comprising 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropylsilane (TIPS) and 2.5% (v/v) H_2O and allowed to incubate for 2 h at room temperature. The filtrate was collected and concentrated under reduced pressure and mixed with a tenfold excess volume of cold diethyl ether followed by centrifugation (4,000 ×g). Supernatant was removed, and the crude peptide precipitate was dissolved in 10 mL of a 1:1 mixture of acetonitrile (ACN)/H_2O and lyophilized. The cyclic peptide was then purified via semipreparative HPLC using a linear gradient of 5% to 60% acetonitrile in water (with 0.1% TFA) over 30 min. The correct peptide mass was confirmed via MALDI-TOF-MS analysis using CHCA as the matrix (Figure S3). Subsequently, pure fractions were combined and lyophilized. Finally, for removal of TFA, peptides were resuspended in 100 mM HCl and lyophilized to yield the peptide hydrochloride salts.

Synthesis of O4bbY-*i*/*i*-(8/10) Macrocyclic Peptides

Synthesis was carried out by conventional Fmoc-solid-phase peptide synthesis on Knorr amide resin (0.4 mmol g^{-1} on a 0.05-mmol scale). Peptides were generated manually in syringes equipped with a Teflon frit. Initially, the resin was subjected to swelling for 30 min in 6 ml of a 1:1 mixture of dichloromethane/*N*,*N*-dimethylformamide (DCM/DMF). Swollen resin was then treated with 20% piperidine in DMF containing 0.05 M 1-hydroxybenzotriazole (3 cycles of 3 min, 5 min, and 3 min) to achieve deprotection of the Fmoc group. After washing with DMF (5×), the

resin was loaded with eight equiv. of Fmoc-Gly-OH, eight equiv. of HCTU and sixteen equiv. of N, N-diisopropylethylamine (DIPEA) in DMF with respect to the initial loading of the resin. The coupling reaction was allowed to proceed at room temperature for 40 min, followed by washing with DMF (5×). The Fmoc deprotection was performed as previously described and, after washing with DMF (5×), the resin was loaded with 4 equiv. of dipeptide **6** (Scheme S1), 4 equiv. of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluoro-phosphate (HATU) and 8 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 1 h, followed by washing with DMF (5×). The resin was loaded with 20 eq. of acetic anhydride and 40 eq. of DIPEA in DMF to cap any unreacted N-terminal amine groups, and the acetylation reaction was allowed to proceed for 30 min. Upon washing with DMF (5x), Fmoc groups were deprotected, and the resin was washed with DMF (5×). The resin was loaded with 4 equiv. of amino acid, 4 equiv. of HCTU, and 8 equiv. of DIPEA in DMF and the coupling reaction was allowed to proceed for 40 min. The resin was then washed with DMF (5×) and this process was repeated to attain the desired peptide sequence. After coupling the final amino acid of the desired peptide sequence, the Fmoc group was deprotected with 20% piperidine in DMF containing 0.05 M 1-hydroxybenzotriazole as previously described. Next, the resin was treated with a mixture of 20 equiv. of phenyl silane ($PhSiH_3$) and 0.35 equiv. of tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) dissolved in 2.5 mL of DCM and incubated for 1 h with gentile shaking to promote the deprotection of the allyl group. The resin was then washed with DCM (7×) and DMF (4×). Peptide cyclization via amide bond formation was carried out using a mixture of 2.5 equiv. of HATU and 5 equiv. of DIPEA dissolved in 2 mL DMF and shaken overnight at room temperature. The resulting macrocyclic peptide was cleaved from the resin as follows: the resin was washed with DMF (7×), DCM (3×) and dried *in vacuo*. The dried resin was

taken up in 10 mL of cleavage solution comprising 95% (v/v) TFA, 2.5% (v/v) TIPS and 2.5% (v/v) H_2O and allowed to incubate for 2 h at room temperature. The filtrate was collected and concentrated under reduced pressure and mixed with a tenfold excess volume of cold diethyl ether followed by centrifugation (4,000 × g). Supernatant was removed, and the crude peptide precipitate was dissolved in 5 mL of a 1:1 mixture of ACN/H_2O and lyophilized. The cyclic peptide was then purified via semipreparative HPLC using a linear gradient of 10% to 55% acetonitrile in water (with 0.1% TFA) over 30 min. The correct peptide mass was confirmed via MALDI-TOF-MS analysis using CHCA as the matrix (Figure S3). Subsequently, pure fractions were combined and lyophilized. Finally, for removal of TFA, peptides were resuspended in 100 mM HCl and lyophilized to yield the peptide hydrochloride salts.

Recombinant Expression and Purification of Macrocyclic Peptides

Macrocyclic peptides were produced recombinantly in accordance with previously reported procedures.[1, 2, 4] Initially, a fusion construct for **FSS-m1** was designed in the following order: N-terminal FLAG-tag, the macrocyclic precursor sequence, Factor Xa cleavage site, chitin-binding domain (CBD) and a C-terminal His-tag. Briefly, the gene encoding the fusion construct was amplified by PCR with forward primer **24** and reverse primer **23** using a previously described pET22 template vector containing an N-terminal FLAG-tag, Factor Xa cleavage site, CBD and a C-terminal His-tag[2]. Subsequently, the PCR product was inserted between the *Bam*HI and *Xho*I restriction sites of the aforementioned construct. Later, a fusion construct for **FSS-m1** was designed to consist of the following: N-terminal Met-Gly leader, the macrocyclic precursor sequence, GyrA intein and a C-terminal His-tag. The gene encoding the GyrA-fusion construct was amplified by PCR using forward primer **26** and reverse primer **25** using a different, previously

described GyrA-containing pET22 construct as the template[4]. Subsequently, the PCR product was inserted between the *Nde*I and *Xho*I restriction sites of the same GyrA-containing vector. Plasmids for the expression of the alanine scanning variants were prepared by substituting each residue of the macrocyclic precursor sequence individually with Ala via site-directed mutagenesis using the parent GyrA-containing **FSS-m1** fusion construct as the template, forward primers **27**–**33** and reverse primer **26**. All recombinant peptides were produced in *E. coli* BL21(DE3) cells via co-transformation of the appropriate expression plasmid and pEVOL-TyrRS42, an amber suppressor plasmid that has been demonstrated previously to incorporate pCaaF upon readthrough of the amber stop codon[2]. *E. coli* BL21(DE3) cells containing both an expression plasmid and a suppressor plasmid were grown overnight in 5 mL LB media supplemented with ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Overnight cultures then were used to inoculate 1 L of fresh LB media supplemented with ampicillin and chloramphenicol at 37 °C. Upon reaching an OD_{600} of 0.6, the temperature was reduced to 27 °C, and cells were induced with arabinose (0.06% m/v) and supplemented with pCaaF (2 mM). After 1 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added, and cells were grown overnight. The following day, cells were harvested by centrifugation (4,000 x g), lysed via sonication, and clarified by centrifugation (10,000 x g). Peptides were purified by Ni-NTA affinity chromatography and buffer exchanged with phosphate buffered saline (PBS) three times. Subsequently, for cleavage of the GyrA intein, peptides were incubated in a solution of PBS containing 20 mM tris(2-carboxyethyl)phosphine (TCEP) and 10 mM thiophenol at pH 8.5 overnight at room temperature. Then, the cleaved peptides (i.e., MG-**FSS-m1** and its alanine variants) were purified via solid phase extraction with a step gradient of acetonitrile in water (+0.1% TFA). Upon confirmation of peptide identities by MALDI-TOF-MS,

pure fractions were combined and lyophilized. Subsequently, for removal of TFA, peptides were resuspended in 100 mM HCl and lyophilized to yield the final peptide hydrochloride salts.

Surface Plasmon Resonance

The biotinylated SARS-CoV-2 RNAs (Figure 2) were generated by chemical synthesis (Horizon Discovery) and deprotected and desalted prior to use. The RNA was folded as described above then diluted in folding buffer to create a 1 μM stock. The RNA was immobilized on CM5 chips (Cytiva) conjugated to Neutravidin to achieve ~1000 RU using an SPR buffer comprising 0.010 M HEPES pH 7.0 and 0.15 M NaCl. Similarly, a 200 nM stock of biotinylated SARS-CoV-2 dimerization loop diluted in SPR buffer was flowed over the chip surface to achieve 1700 RU. Analysis of pCaaF(*i*-10)-m1 (**FSS-m2**) proceeded in SPR buffer. Analysis of the related peptide pCaaF(*i*-8)-m1 (**FSS-m1**) and all recombinant peptides proceeded in a 2× salt SPR buffer (0.010 M HEPES pH 7.0 and 0.30 M NaCl). O4bbY/Cys peptides (i.e., *i*/*i*+8 or *i*/*i*+10) were analyzed in an SPR buffer comprising 0.010 M HEPES pH 7.0, 0.15 M NaCl and 0.05% Tween 20, while Cys/O4bbY peptides (i.e., *i*/*i*-8 or *i*/*i*-10) were analyzed in an SPR buffer comprising 0.010 M HEPES pH 7.0 and 0.15 M NaCl without Tween 20. The flow rate for each experiment was 30 μL/min. Cyclic peptides at various concentrations (1.9–30.0 μM for pCaaF family peptides and 1.3–20.0 μM for O4bbY family peptides) were injected for 60 s and allowed to dissociate for 180 s. Similarly, recombinant peptides at a concentration of 2.5 μM were injected for 60 s and allowed to dissociate for 180 s. To regenerate the RNA, 2 M NaCl was injected for 60 s for pCaaF family peptides, and 3 M guanidine HCl was injected for 60 s for O4bbY family peptides. To reduce nonspecific binding during peptide assays, a 100-fold molar excess of yeast tRNA with respect to the immobilized RNA was added to each SPR buffer and the experiments were repeated for each

peptide. Experimental data were processed using the double-referencing method[5]. The buffer-subtracted sensorgrams were fit to a 1:1 binding model using Biacore T200 analysis software to determine rate constants (k_{on} and k_{off}) and the apparent equilibrium dissociation constant (K_D)[6]. The results were plotted using Prism software (GraphPad Inc.). The K_D for the equilibrium binding measurements was determined by taking the average response from a 5 s window at equilibrium (R_{eq}) for each peptide injection versus the peptide concentration using Prism software; data were then fit to a one-site binding model. The kinetic and equilibrium experiments were repeated in triplicate with the exception of O4bbY(*i*-10)-m1 (**FSS-m8**), which was repeated in duplicate.

Biotinylated RNAs chosen for off-target binding analysis were produced by chemical synthesis and deprotected by the manufacturer followed by desalting (Horizon Discovery). Each RNA corresponded to a well-characterized riboswitch. We tested the following sequences: (i) the 33-mer class I type II preQ$_1$ riboswitch from *Thermoanaerobacter tengcongensis* (*Tte*), which binds a single equivalent of preQ$_1$ with a K_D of 2.1 ± 0.3 nM;[6] (ii) the 34-mer class I type I preQ$_1$ riboswitch from *Carnobacterium antarticum* (*Can*), which cooperatively binds two preQ$_1$ equivalents in the same pocket with a K_{D1} of 891 nM and K_{D2} of 461 nM;[7] and a 64-mer guanine-I riboswitch variant from *Paenibacillus pectinilyticus* (*Ppe*), which binds a single equivalent of guanine with a K_D of approximately 351 ± 99 nM.[8] The corresponding sequences are: (i) *Tte* riboswitch CUGGGUCGCAGUAACCCCAGUUAACAAAACCCG; (ii) *Can* riboswitch UGUGGUUCGCAACCAUCCCACAUAAAAAAACUAG; and (iii) *Ppe* riboswitch GGCCGUAUAACCUCGAUAAUUUGGUUCGGGGGCUCUACUGGGAACCUAAAUCCU AACUACGGCC. The *Tte* and *Can* strands were dissolved in 0.01 M Na-cacodylate pH 7.0; strands were folded by heating to 90 °C for 2 min. Sample tubes were rapidly cooled on ice, and an equal volume of 0.1 M Na-cacodylate containing 0.006 M $MgCl_2$ was slowly added followed

by a 30 min incubation on ice prior to warming to room temperature for SPR analysis. The *Ppe* riboswitch was folded by dissolving the RNA in 0.01 M Na-HEPES pH 7.0, followed by heating to 80 °C for 2 min, then cooling for 2 min at room temperature, followed by the slow addition of an equal volume of 0.01 M Na-HEPES pH 7.0, 0.1 M KCl, and 0.01 M $MgCl_2$. Subsequently, the riboswitch was placed on ice for at least 30 min prior to warming to room temperature for SPR. Each folded riboswitch stock was then diluted in the SPR running buffer to 300 nM prior to immobilization on CM5 chips with a neutravidin surface. The cyclic peptides (**FSS-m1** & **FSS-m2**) were analyzed in an SPR buffer comprising 0.010 M HEPES pH 7.0, 0.30 M NaCl and 0.05% Tween 20 that was passed over surfaces of each immobilized riboswitch (~800–1500 RU). The flow rate, association and dissociation conditions were the same as those used for the biotinylated SARS-CoV-2 RNAs SPR experiments (above). Cyclic peptide concentrations of 6.25 or 12.5 μM were injected and passed over the chip, which is approximately 3.1 to 6.3-fold higher than the K_D measured for **FSS-m1** and **FSS-m2** binding to the FSS-PK (Table 2) and 18–6000-fold greater than the affinity of each riboswitch for its natural ligand; 3 M guanidine HCl was injected twice for 90 s at a flow rate of 60 μL/min to regenerate the riboswitch surfaces.

Cell Viability Assay

Cell viability was determined using HEK293T cells (ATCC, CRL-3216) (a gift from Prof. Sheel Dodani) seeded in 24-well plates (Falcon), and the cells were stored in a 37 °C humidified incubator with 5% CO_2. Per manufacturer's recommendations (ATCC), HEK293T cells were plated at a density of 1 x 10^4 to 4 x 10^4 viable cells/cm^2 in 500 μL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11995065) supplemented with 10% fetal bovine serum (FBS) (Corning, 35011CV) and 1% penicillin/streptomycin (Gibco, 15140122). Once the cells reached a

confluency of 70–80%, the **FSS-m1** cyclopeptide was diluted from a stock solution into the aforementioned culture media and incubated with HEK293T cells to yield final concentrations of 2 μM, 10 μM and 50 μM each with 1% (v/v) dimethyl sulfoxide (DMSO). Similarly, parthenolide (PTL), a natural product which served a positive control, was diluted into the culture media from a DMSO stock solution and added to the cells at a fixed dose of 50 μM with 1% DMSO. As negative controls, culture media without cyclopeptide or PTL was incubated with HEK293T cells either in the presence or absence of DMSO (final concentration of 1%). Following 24 h of incubation, culture media was removed swiftly and discarded. Subsequently, 500 μL of thiazolyl blue tetrazolium bromide (MTT) solution (1 mg/mL) from a light-protected tube was added to the cells. As a comparator, 500 μL of the aforementioned MTT solution was added to a separate 24-well plate in parallel. Following the addition of the MTT solution, the plates were wrapped in aluminum foil and stored in the humidified incubator. Upon incubation at 37 °C for 3 h, the plates were centrifuged (3,400 x g) for 15 min, the media was removed, and 500 μL of DMSO was added to solubilize the formazan product. The absorbance of the samples was measured at a wavelength of 550 nm using a multi-well plate reader (Agilent). Analyses were conducted in triplicate for cells treated with DMSO only and 10 μM **FSS-m1**, while analyses were conducted in quadruplicate for all other cells (i.e., untreated without DMSO, 50 μM PTL treatment, 50 μM **FSS-m1** treatment and 2 μM **FSS-m1** treatment).

Selective 2′ Hydroxyl Acylation Analyzed by Primer extension (SHAPE)-Seq

For chemical-modification experiments, the dimerization loop was synthesized by T7 polymerase.[9] The transcription product contained the 26-mer dimerization loop of the SARS-CoV-2 FSS placed in a folding cassette. The 5′-end of the cassette possessed the stable HIV-1 FSS stable

hairpin, whereas the 3′-end harbored a strong hairpin followed by a unique primer binding site as described.[10] Additionally, the HIV-1 FSS serves as a control for the specificity of the peptides for the SARS-CoV-2 FSS. The 17-mer DNA primer and DNA template were ordered from IDT Inc. The transcript synthesized by T7 polymerase was:

5′-GGCTTCCCACAAGGGAAGCCATGGCGGCACAGGCACTAGTACTGAT

GTCGCCTCGATCCGCTTCGGCGGATCCAAATCGGGCTTCGGTCCGGTTC-3′. The *in vitro* transcribed product was PAGE purified, desalted[9] and stored at −20 °C in 0.5× TE buffer.

Prior to modification, 60 pmol of pure RNA was dissolved in 72 μL of 0.5× TE buffer per modification condition. The RNA was heated to 95 °C for 2 min and placed on ice 2 min. A 36 μL volume of 3× RNA folding mix (333 mM HEPES pH 8.0, 333 mM NaCl and 20 mM $MgCl_2$) was added; for the bound-state conditions, peptide was also added to a final concentration 10× the K_D of the cyclic peptide for the RNA as determined by SPR. The RNA was incubated at 24 °C for 20 min.

The 72-μL-folded-RNA sample was split in half after the addition of 36 μL of folding mix, yielding 18 μL of free-state or bound-state RNA per reaction condition. Acylation was performed with 2-methylnicotinic acid imidazolide (NAI) (Sigma-Aldrich).[11] A volume of 6 μL of 0.6 M NAI in DMSO was added to a concentration of 150 mM. Alternatively, 6 μL of DMSO was added to the control samples. The reactions proceeded 15 min at 24 °C and were terminated by addition of a stop solution comprising 90 μL water, 5 μL 4 M NaCl, 1.5 μL GlycoBlue and 2 μL of 100 mM EDTA pH 8.0. The RNA was ethanol-precipitated by addition of 350 μL of neat ethanol. Precipitated RNA was harvested by centrifugation at 18000 ×g for 40 min at 4 °C. The pellet was washed in 350 μL cold neat ethanol, and re-harvested by centrifugation at 18000 ×g for 40 min at 4 °C. The air-dried pellet was dissolved in 20 μL of 0.5× TE buffer.

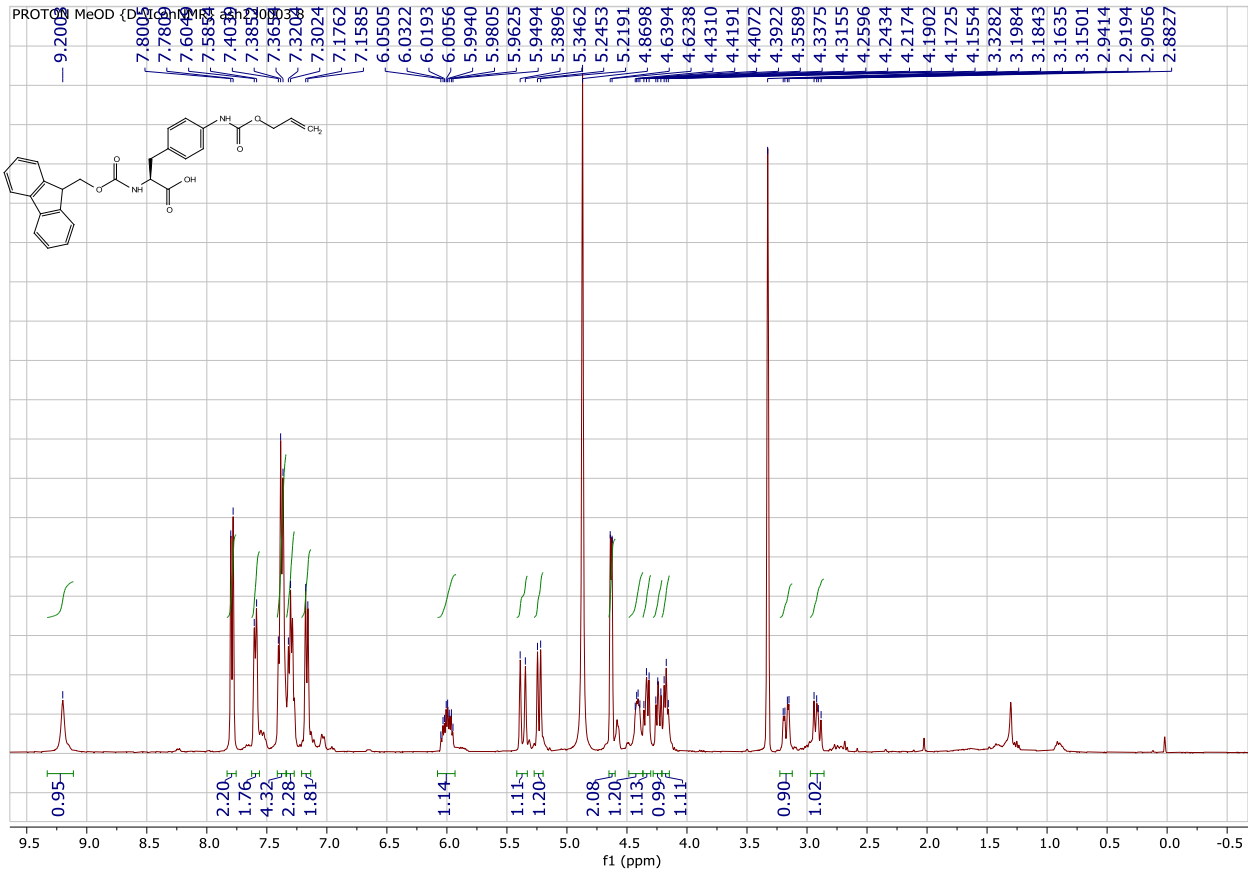
Modification of RNA was analyzed using the SHAPE-seq v2.1 workflow[12] as described[13] with an additional purification step where samples were size selected to using a 2% agarose gel on the PippinHT system (Sage Science, MA). Each sample was analyzed with SPATS version 1.9.30.[14] SHAPE reactivities were calculated using the following target sequence: GGCTTCCCACAAGGGAAGCCATGGCGGCACAGGCACTAGTACTGATGTCGCCTCGA TCCGCTTCGGCGGATCCAAATCGGGCTTCGGTCCGGTTC.

Calculation of logP values

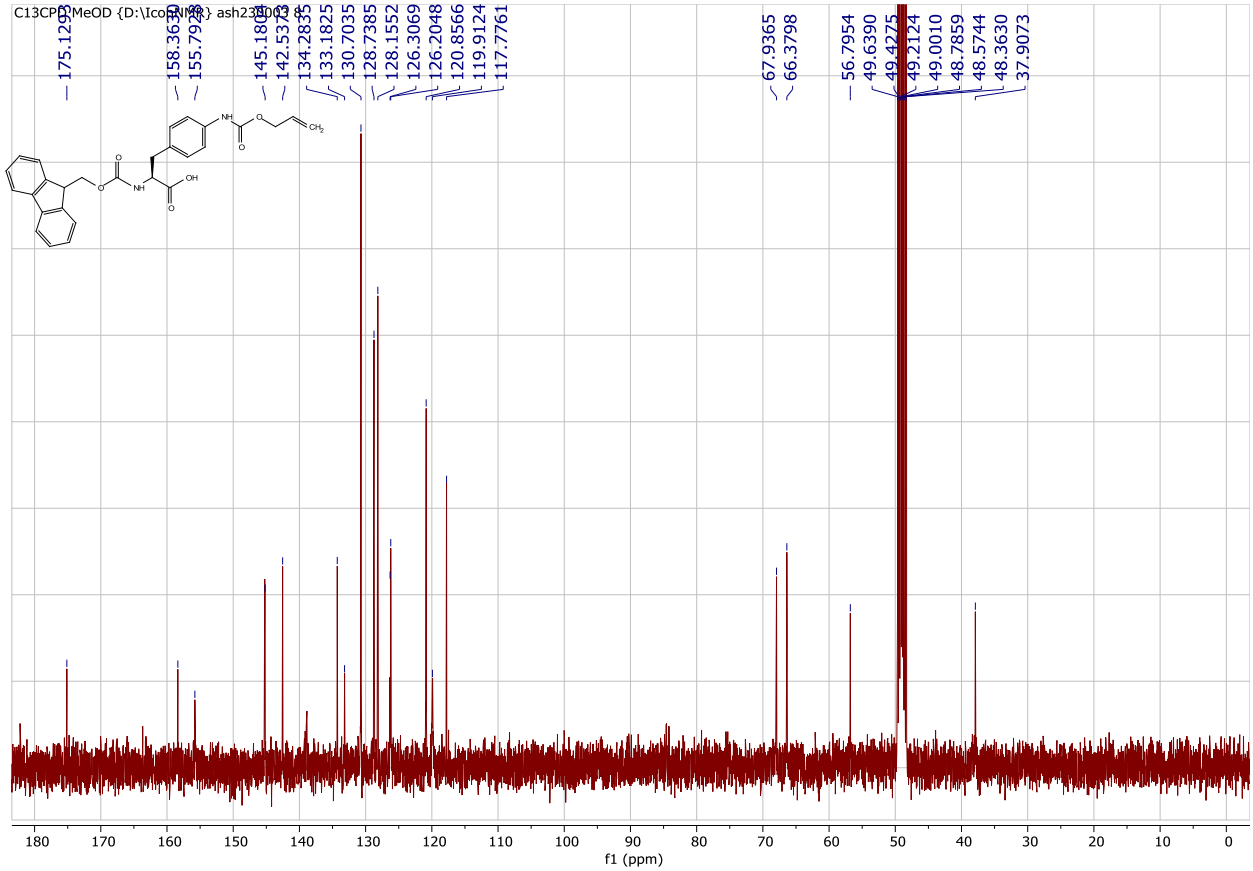
Cyclic peptides were converted into SMILES strings using ChemDraw (Revvity Signals). For each cyclic peptide, the SMILES string was copied into the program AloGPS v2.1,[15] and the logP value was calculated using the non-JAVA-enabled web tool: https://vcclab.org/web/alogps/.

NMR Spectra

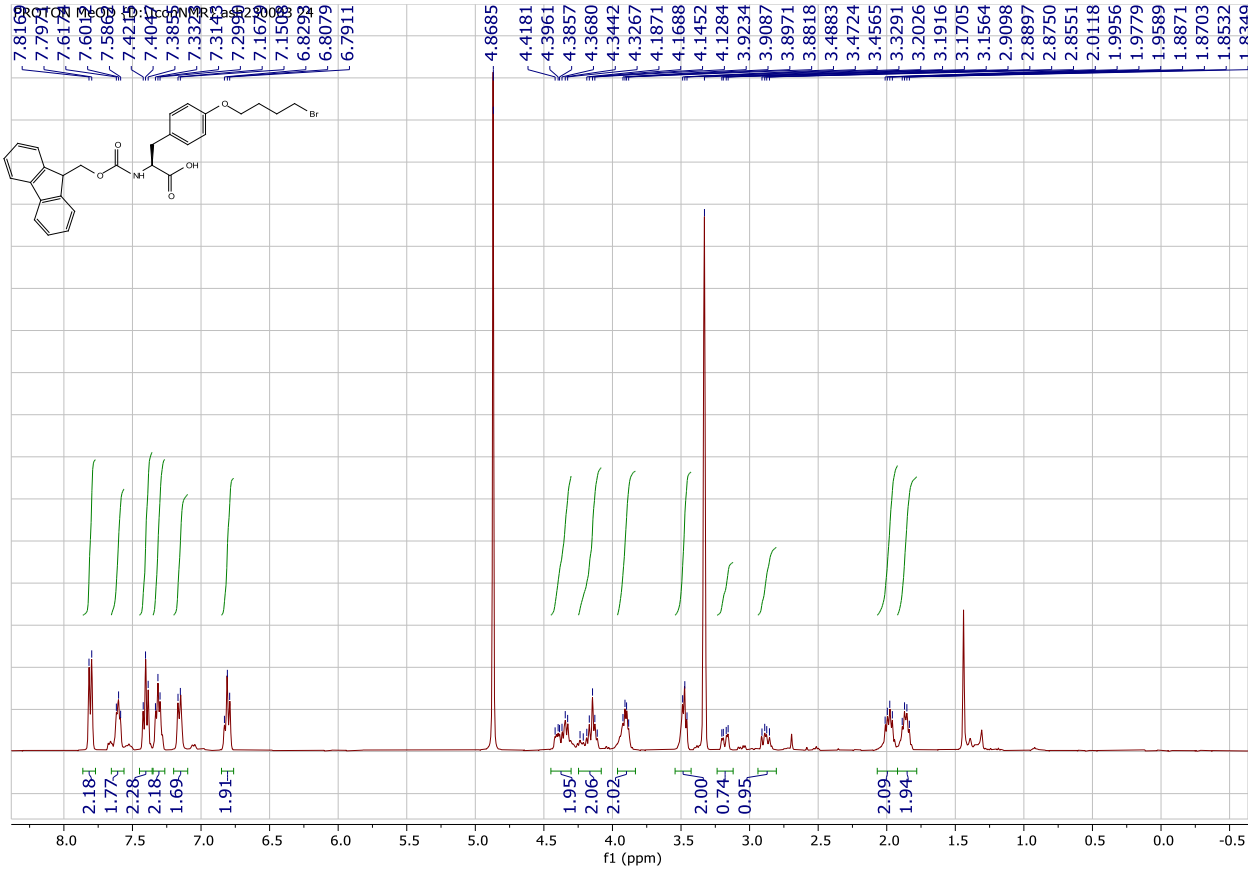
(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(((allyloxy)carbonyl)-amino)phenyl)propanoic acid (N-Fmoc-N´-Alloc-*p*-amino-L-Phe-OH)



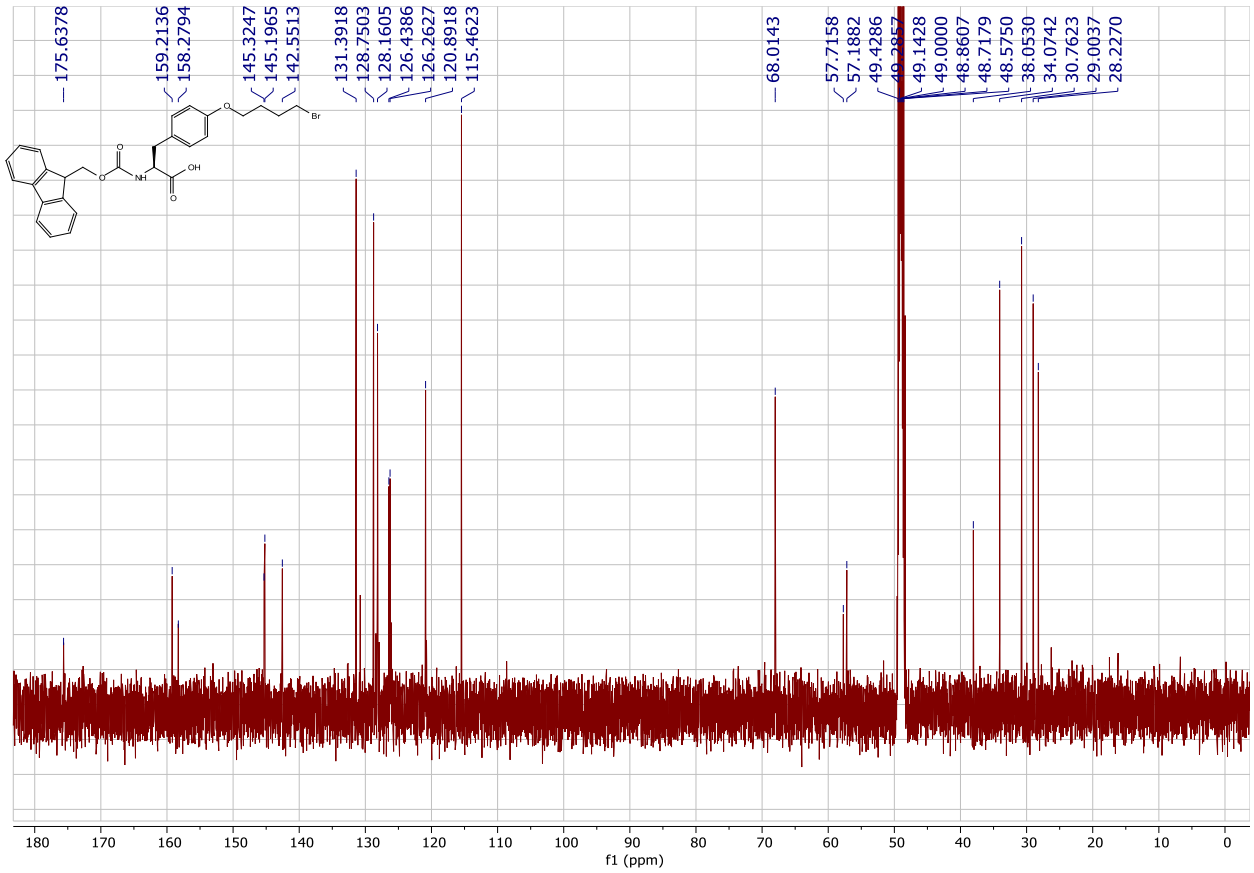
(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(((allyloxy)carbonyl)-amino)phenyl)propanoic acid (N-Fmoc-N´-Alloc-*p*-amino-L-Phe-OH) (cont.)



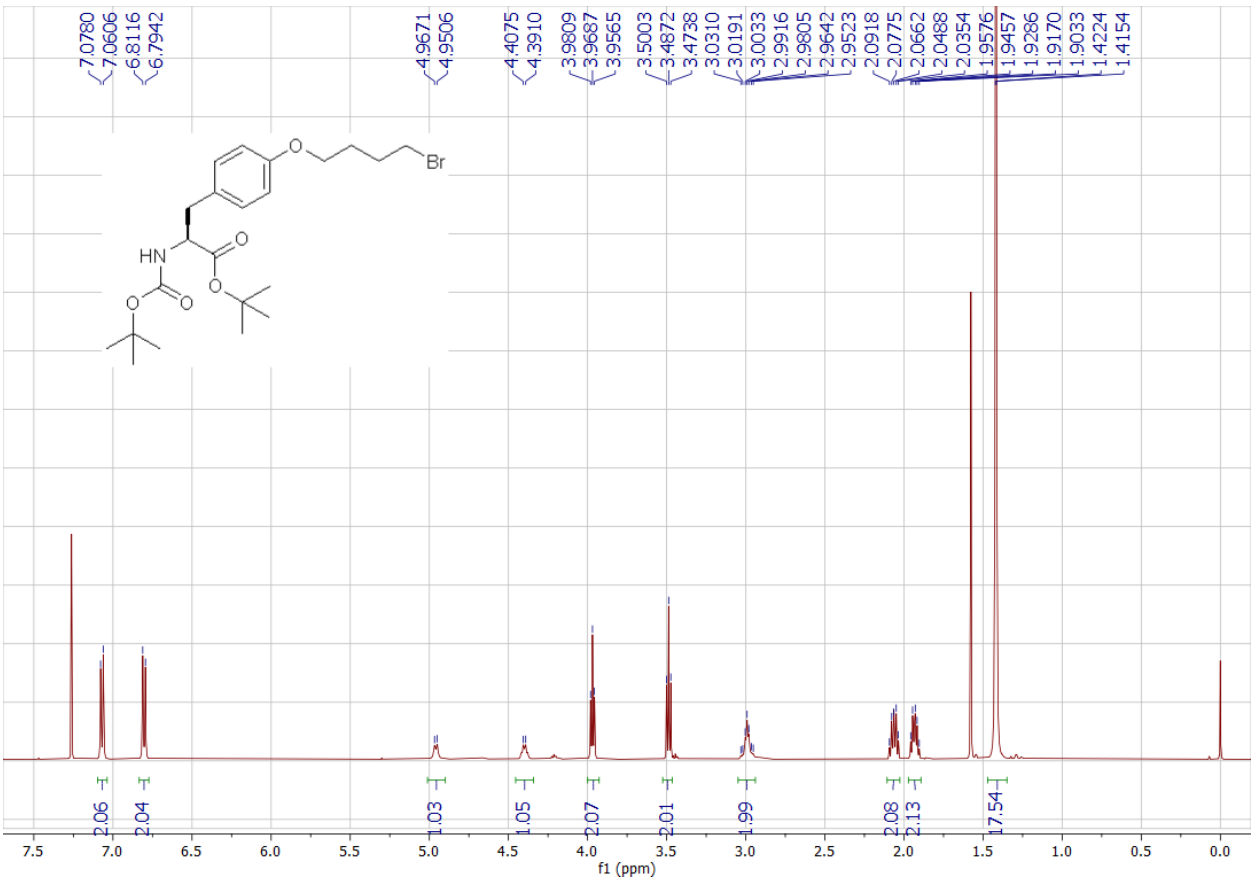
(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-bromobutoxy)-phenyl)propanoic acid (Fmoc-O4bbY-OH)



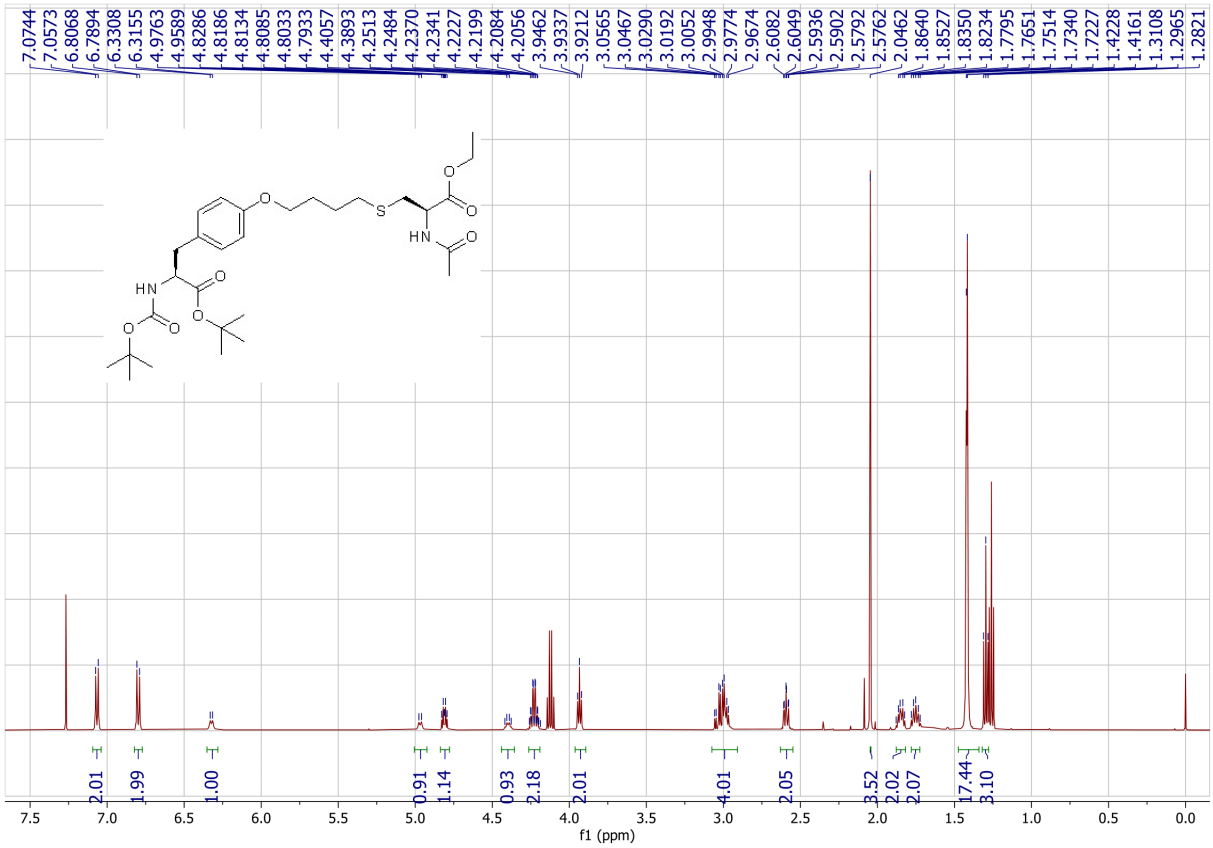
(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-bromobutoxy)-phenyl)propanoic acid (Fmoc-O4bbY-OH) (cont.)



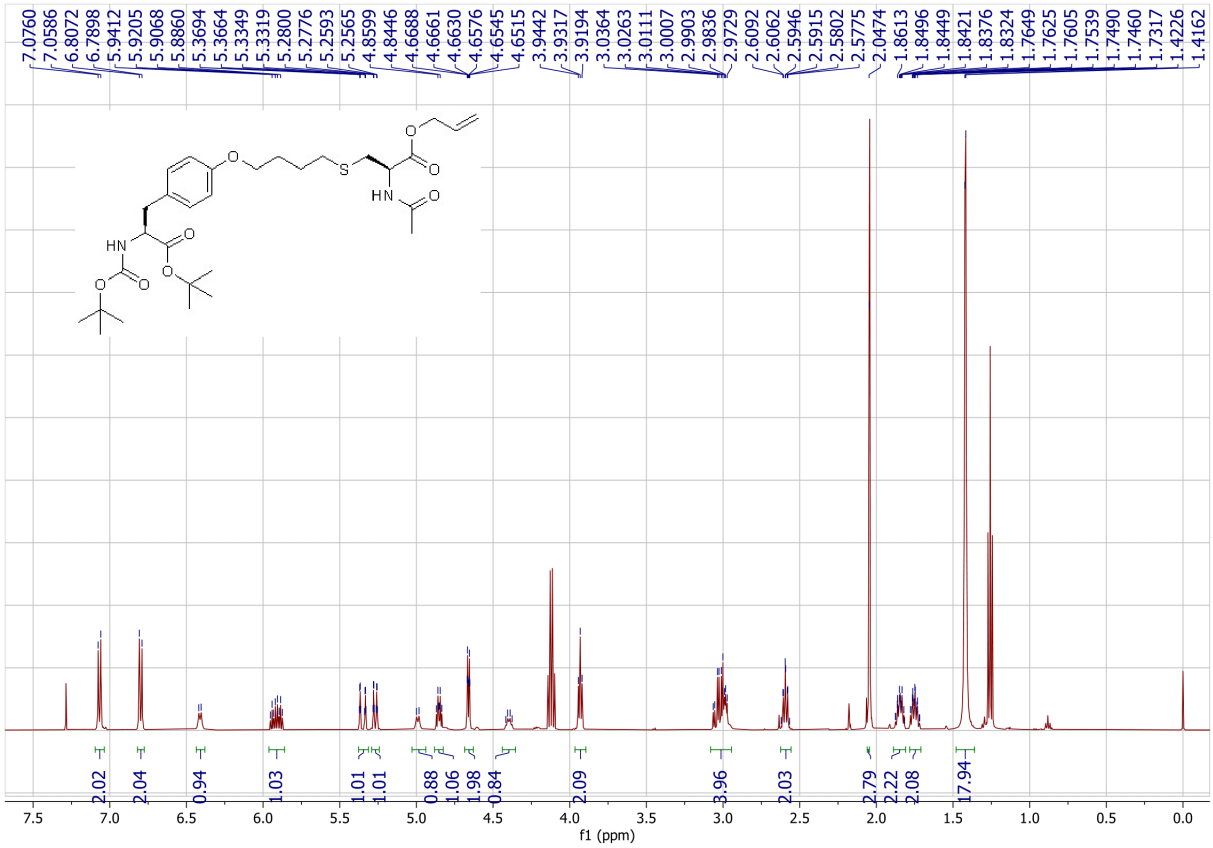
_t_-Butyl (_S_)-3-(4-(4-bromobutoxy)phenyl)-2-((_tert_-butoxycarbonyl)amino)propanoic acid (2)



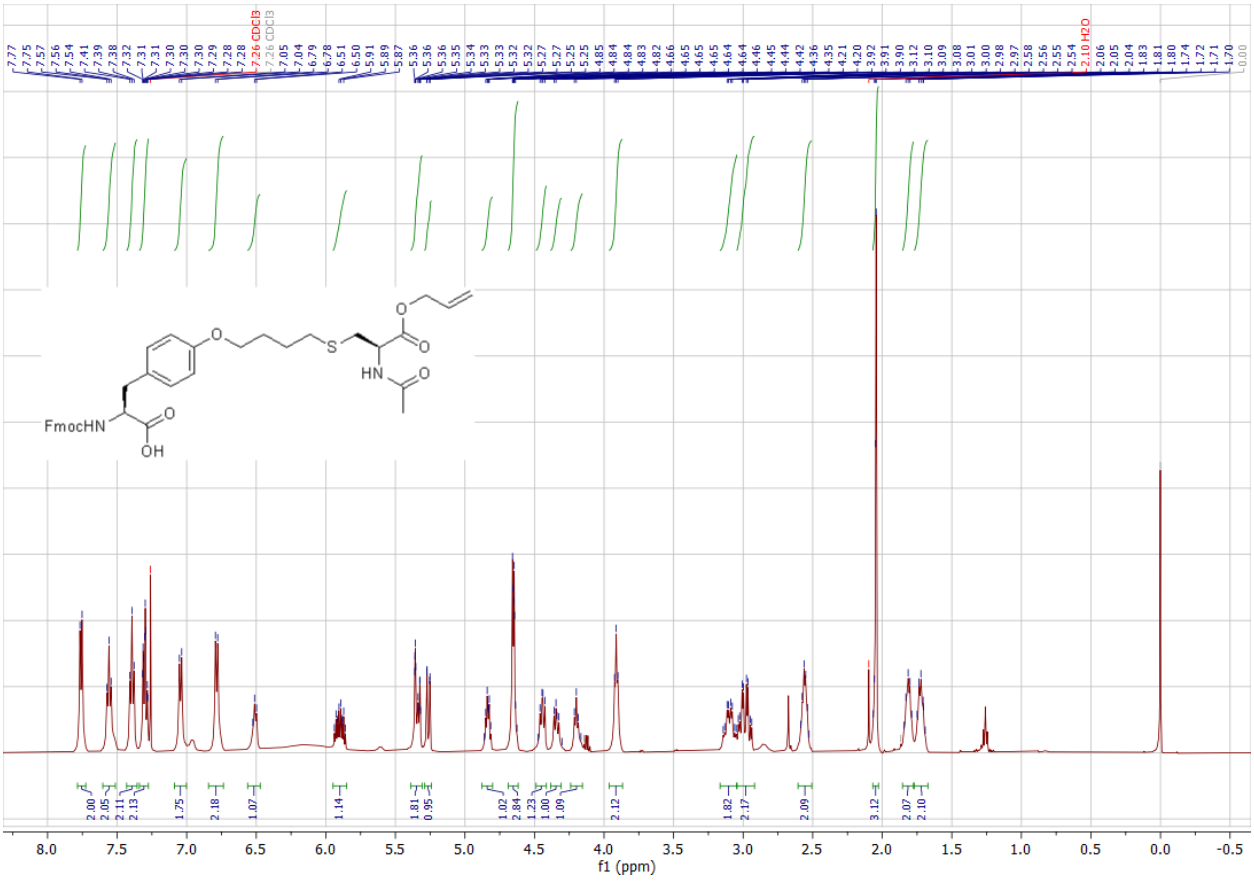
***t*-Butyl (*S*)-3-(4-(4-(((*R*)-2-acetamido-3-ethoxy-3-oxopropyl)thio)butoxy)phenyl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (4)**



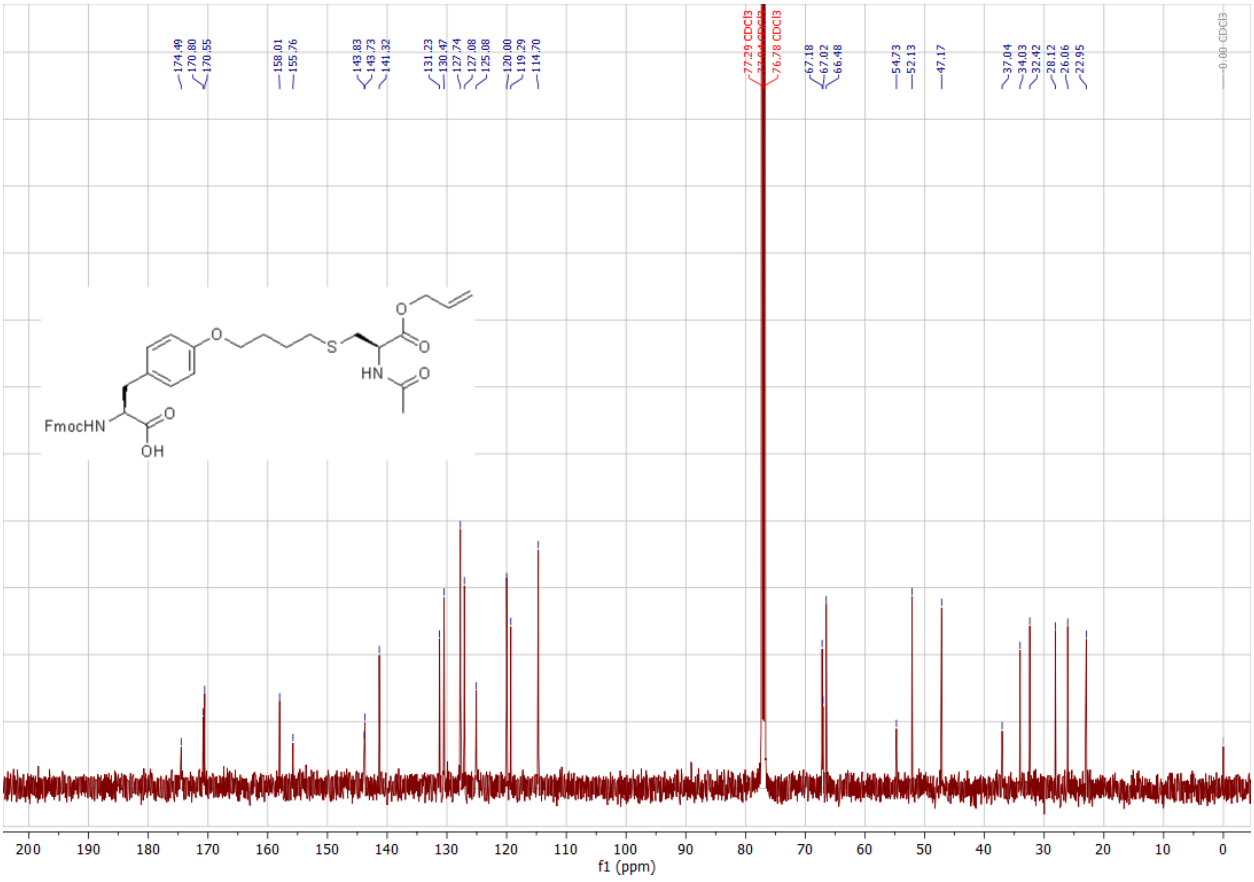
Allyl *N*-acetyl-*S*-(4-(4-((*S*)-3-(tert-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)-phenoxy)butyl)-L-cysteine (5)



(*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-(((*R*)-2-acetamido-3-(allyloxy)-3-oxopropyl)thio)butoxy)phenyl)propanoic acid (6)



(*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(4-(((*R*)-2-acetamido-3-(allyloxy)-3-oxopropyl)thio)butoxy)phenyl)propanoic acid (6) (cont.)



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