Supplementary Information

Discovery of macrocyclic peptide binders, covalent modifiers, and degraders of a structured RNA by mRNA display

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Materials and methods:

Table S1. Materials used for mRNA display and synthesis.

REAGENT	SOURCE	IDENTIFIER
mRNA display		
MEGAscript T7 Transcription Kit	Invitrogen	AM1334
Q5 High-Fidelity DNA Polymerase	New England Biolabs	M0491L
BSA, acetylated (20 mg/mL)	Invitrogen	AM2614
PowerUp SYBR Green Master Mix for qPCR	Applied Biosystems	A25777
PURExpress Δ RF123 Kit	New England Biolabs	E6850S
SUPERase·In RNase Inhibitor (20 U/μL)	Invitrogen	AM2696
Low Range ssRNA Ladder	New England Biolabs	N0364S
Pierce Streptavidin Magnetic Beads	Thermo Scientific	88816
RNA Clean & Concentrator-25	Zymo Research	R1018
Dynabeads Oligo$(dT)_{25}$	Invitrogen	61005
Lysis/Binding Buffer for Dynabeads mRNA Purification Kits	Invitrogen	A33562
Wash Buffer B for Dynabeads mRNA Purification Kits	Invitrogen	11900D
Pierce Anti-HA Magnetic Beads	Thermo Scientific	88837
HA Peptide (YPYDVPDYA)	GenScript	RP11735
T4 RNA Ligase 1 (ssRNA Ligase)	New England Biolabs	M0204L
ZR small-RNA PAGE Recovery Kit	Zymo Research	R1070
$MgCl_2$ (1 M)	Invitrogen	AM9530G
KCl (2 M), RNase-free	Invitrogen	AM9640G
Tween-20	Sigma Aldrich	P1379
PrimeScript™ RT Reagent Kit	Takarabio	RR037B
Qubit dsDNA HS Assay Kit	Invitrogen	Q32851
TAE Buffer (Tris-acetate-EDTA) (50X)	Thermo	B49
TBE Buffer (Tris-borate-EDTA) (10X)	Thermo	B52
Peptide and Synthesis		
Rink Amide resin	Sigma Aldrich	8.55001
HATU	Ambeed	A633512
Fmoc-Ala-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-His(trt)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH	Combi-Blocks	QA-4308, QA-3218, SS-0200, OR-0415, NA-0029, QA-0010, QB-3100, QE-9409, OR-0575, QA-4475, QB-8461, SS-0147, QA-2435, QB-0626, QA-9886, SS-0149, SS-0148, QA-2487, SS-0604, QB-1094
1-Hydroxy-7-azabenzotriazole solution, 0.6 M in DMF	Chem-Impex	14324
3,5-Di(hydroxymethyl)phenol	1ClickChemistry	6C52807
Hydrogen bromide solution, 33 wt. % in acetic acid	Sigma-Aldrich	248630

4-(Acetylamino)phenyl]imidodisulfuryl difluoride	Sigma-Aldrich	901243
3,5-Dimethylbenzenesulfonyl fluoride	Combi-Blocks	QH-7648
N-Bromosuccinimide	Sigma-Aldrich	B81255
2,2′-Azobis(2-methylpropionitrile)	Sigma-Aldrich	441090
tert-butyl 3-(2-(2-(2-(tosyloxy)ethoxy)ethoxy)ethoxy)propanoate	1ClickChemistry	1C43051
Sodium *tert*-butoxide	Sigma-Aldrich	359270
Acetic anhydride	Sigma-Aldrich	539996
Others		
α-Cyano-4-hydroxycinnamic acid	Sigma-Aldrich	70990
Somatostatin	Sigma-Aldrich	S9129
Adrenocorticotropic Hormone Fragment 18-39	Sigma-Aldrich	A8346
dNTP mix	NEB	N0447S
SuperScript™ III Reverse Transcriptase	Invitrogen	18080093
ddNTP Bundle	Jena Bioscience	NU-1019S
Nucleoside Digestion Mix	NEB	M0649S
N-Acetyl-L-histidine monohydrate	Ambeed	39145-52-3
N-Acetyl-L-tyrosine	Ambeed	537-55-3
N-Acetyl-L-arginine dihydrate	Ambeed	210545-23-6
N-Acetyl-L-serine	Combi-Blocks	16354-58-8
Nα-Acetyl-L-lysine	Sigma-Aldrich	1946-82-3

Table S2. List of oligonucleotides used in this study.

Notes	SOURCE	Sequence 5'-3'
Oligo Nucleotides		
SNCA IRE	Horizon	ACUGGGAGUGGCCAUUCGACGACAGUGUGGUGUAAAG GAAUUCAUUAGCC
Base paired *SNCA* IRE	Horizon	ACUGGUGAGUGGCCAUUCGACGCCACAGUGUGGUGUC GAAUGGCCAUUCAUUAGCC
5' biotinylated *SNCA* IRE	Horizon	Bi.ACUGGGAGUGGCCAUUCGACGACAGUGUGGUGUAA AGGAAUUCAUUAGCC
5' biotinylated base paired *SNCA* IRE	Horizon	Bi.CUGGUGAGUGGCCAUUCGACGCCACAGUGUGGUGUC GAAUGGCCAUUCAUUAGCC
5' FITC labeled *SNCA* IRE	Horizon	FI.ACUGGGAGUGGCCAUUCGACGACAGUGUGGUGUAAA GGAAUUCAUUAGCC
5' FITC labeled base paired *SNCA* IRE	Horizon	FI.CUGGUGAGUGGCCAUUCGACGCCACAGUGUGGUGUC GAAUGGCCAUUCAUUAGCC
HIV TAR	Horizon	GGCCAGAUCUGAGCCUGGGAGCUCUCUGGCC
Base paired HIV TAR RNA	Horizon	GGCCAGAUCUGAGCCUGGAGGCUCAGAUCUGGCC
5' biotinylated HIV TAR	Horizon	Bi.GGCCAGAUCUGAGCCUGGGAGCUCUCUGGCC
5' biotinylated base paired HIV TAR	Horizon	Bi.GGCCAGAUCUGAGCCUGGAGGCUCAGAUCUGGCC
5' FITC labeled HIV TAR	Horizon	FI.GGCCAGAUCUGAGCCUGGGAGCUCUCUGGCC
5' FITC labeled base paired HIV TAR	Horizon	FI.GGCCAGAUCUGAGCCUGGAGGCUCAGAUCUGGCC
DNA oligo for ligation	IDT	d(pCCCTTCACCTGATCCGCTGAAAAAAAAAAAAAAAA A)-(iSp18)-(iSp18)-d(FluorT)-(iSp18)-d(CC)-Puromycin
Primers and ultramers		
Forward primer for generating randomized library	IDT	GTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCT CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC AATG
Reverse ultramer for $(NNK)_6$ library	IDT	TTTTTCACCTGATCCGCTGCCTTTCTGTTTGGCGTGGTCCT TGAGGAGCGTATAATCGGGAACATCATAAGGATATGACCC TGAGCCgcaMNNMNNMNNMNNMNNMNNgcaCATTGGTAT ATCTCCTTCTTAAAG
Reverse ultramer for $(NNK)_8$ library	IDT	TTTTTCACCTGATCCGCTGCCTTTCTGTTTGGCGTGGTCCT TGAGGAGCGTATAATCGGGAACATCATAAGGATATGACCC TGAGCCgcaMNNMNNMNNMNNMNNMNNMNNMNNgcaC ATTGGTATATCTCCTTCTTAAAG
Reverse ultramer for $(NNK)_{10}$ library	IDT	TTTTTCACCTGATCCGCTGCCTTTCTGTTTGGCGTGGTCCT TGAGGAGCGTATAATCGGGAACATCATAAGGATATGACCC TGAGCCgcaMNNMNNMNNMNNMNNMNNMNNMNNMNN MNNgcaCATTGGTATATCTCCTTCTTAAAG
Reverse ultramer for $(NNK)_{12}$ library	IDT	TTTTTCACCTGATCCGCTGCCTTTCTGTTTGGCGTGGTCCT TGAGGAGCGTATAATCGGGAACATCATAAGGATATGACCC

		TGAGCCgcaMNNMNNNMNNNMNNNMNNNMNNNMNNNMNN MNNMNNNMNNgcaCATTGGTATATCTCCTTCTTAAAG
Forward primer for PCR	IDT	GTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCT C
Reverse primer for PCR and reverse transcription	IDT	TTTTTCACCTGATCCGCTGCCTTTCTG
Forward primer for qPCR	IDT	GAGACCACAACGGTTTCCCT
Reverse primer for qPCR	IDT	CTTTCTGTTTGGCGTGGTCC
Forward primer for adding sequencing adaptor	IDT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAGACC ACAACGGTTTCCCTCTAG
Reverse primer for adding sequencing adaptor	IDT	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTTCAC CTGATCCGCTGCCTTTCTG
Forward primer for SHAPE RNA template	IDT	TAATACGACTCACTATAGGCGCTGGGAGTGGCCATTCGAC GACAGTGTGGTGTAAAG
Reverse primer for SHAPE RNA template	IDT	CGGCTCATCTTTCAACGGCGCTAATGAATTCCTTTACACC ACACTGTCGTCGAATGG
RT primer for SHAPE	Thermo Fisher	NED-CGGCTCATCTTTCAACG

MALDI-TOF characterization for cyclized peptides (Figure S1-S3)

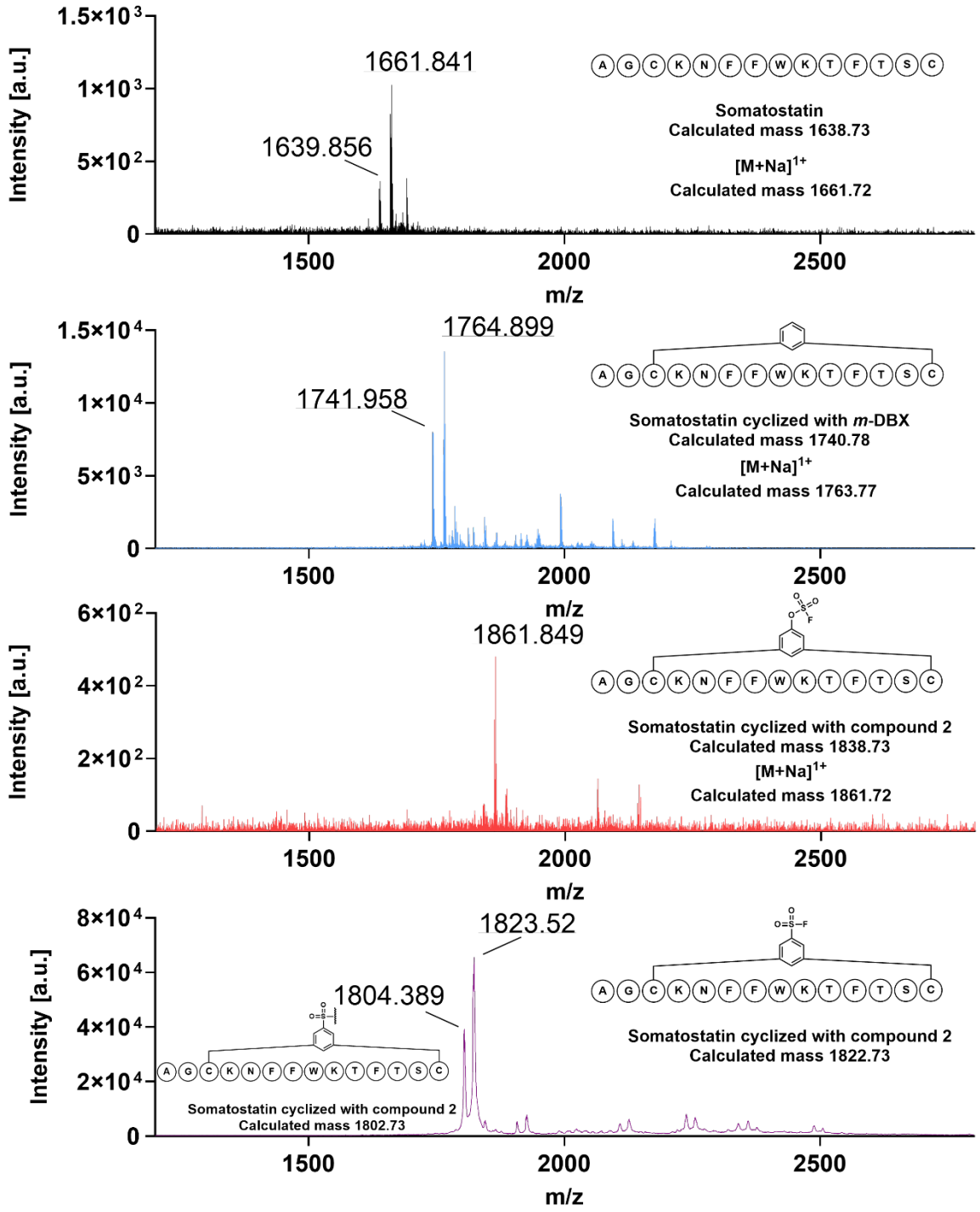


Figure S1. MALDI-TOF characterization of somatostatin cyclized with ***m*-DBX**, **2** and **3**. Reactions were performed in 1X Tris buffer (20 mM Tris HCl, pH 7.8) containing 200 μM TCEP and 30% acetonitrile.

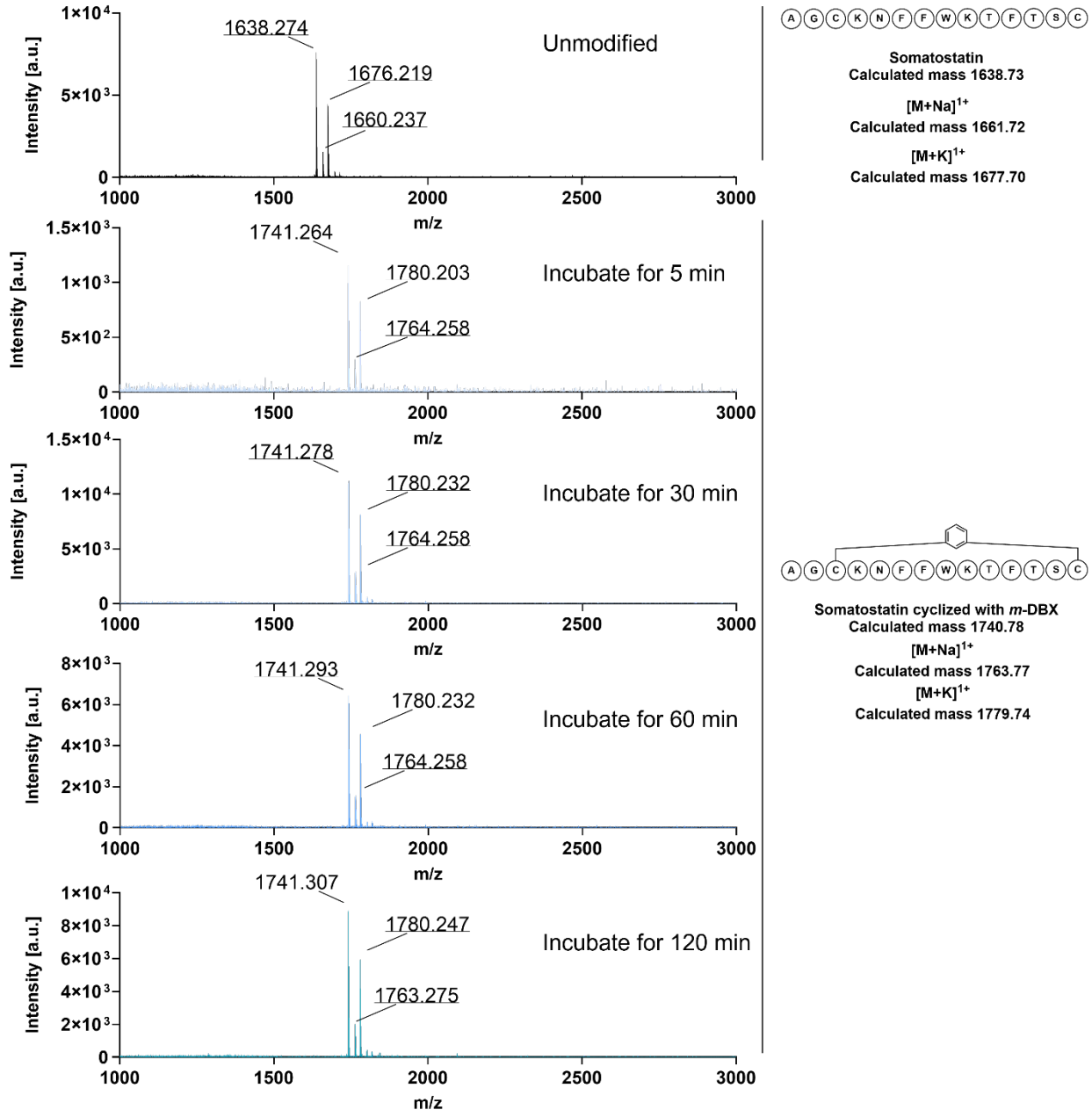


Figure S2. MALDI-TOF analysis of somatostatin cyclization using ***m*-DBX** at varying incubation times.

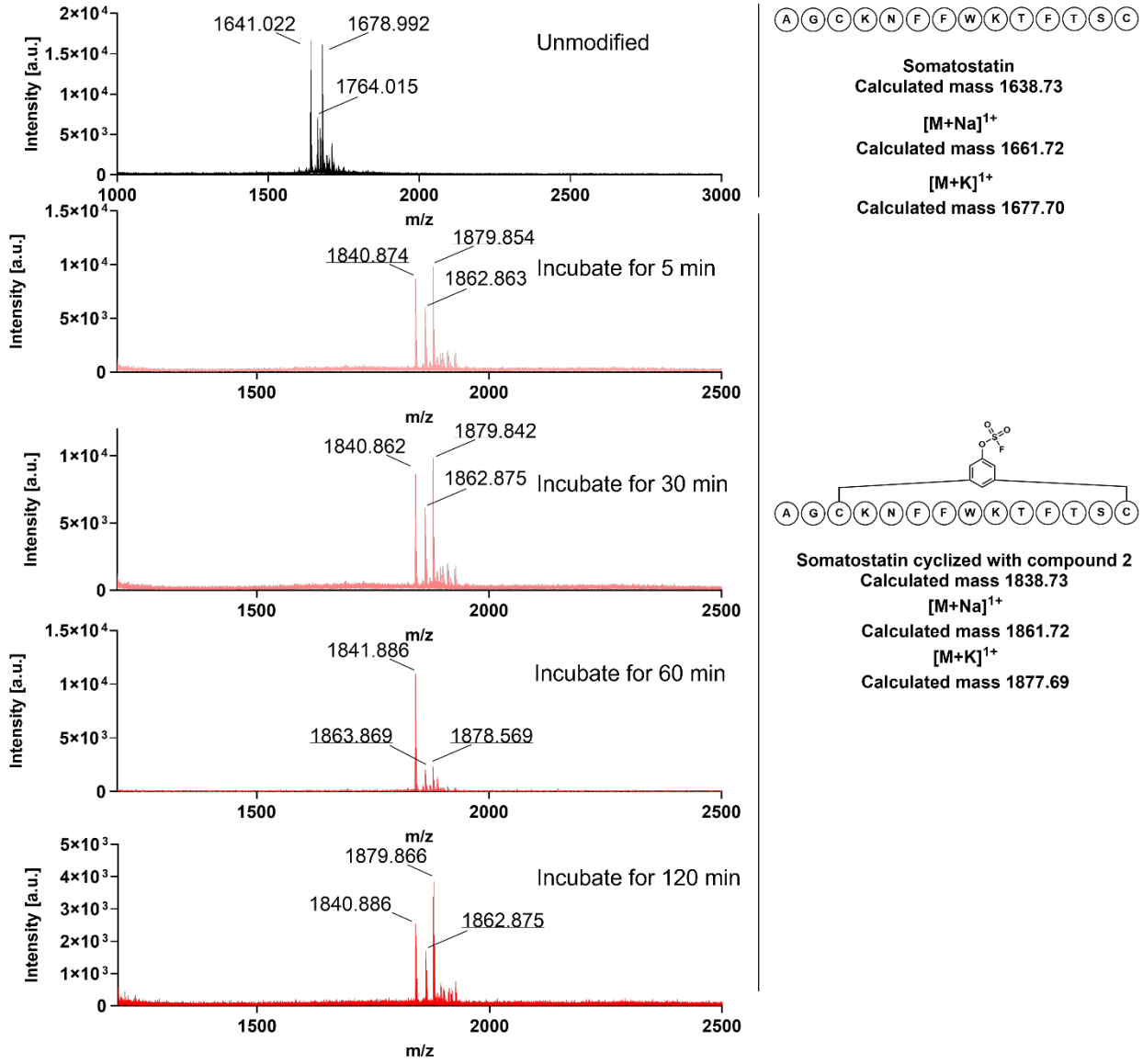


Figure S3. MALDI-TOF analysis of somatostatin cyclization using **2** at varying incubation times.

Binding affinity measurements for HIV TAR hit peptides (Figure S4)

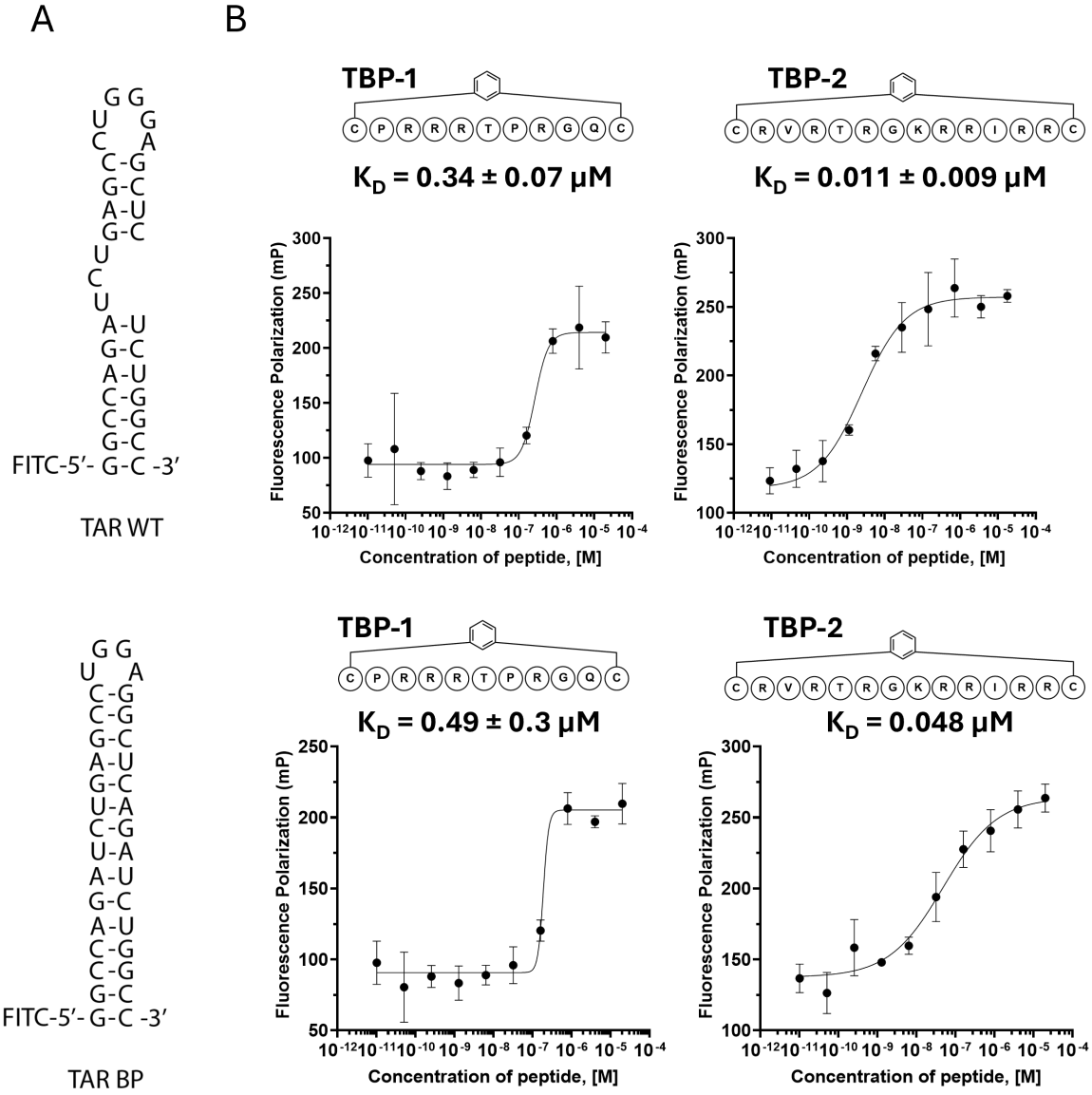


Figure S4. Fluorescence polarization assay for binding analysis of cyclized model peptides, TBP-1 and TBP-2, using HIV TAR (TAR WT) and base-paired control (TAR BP).

Optimization and validations of mRNA display workflow (Figure S5-S8)

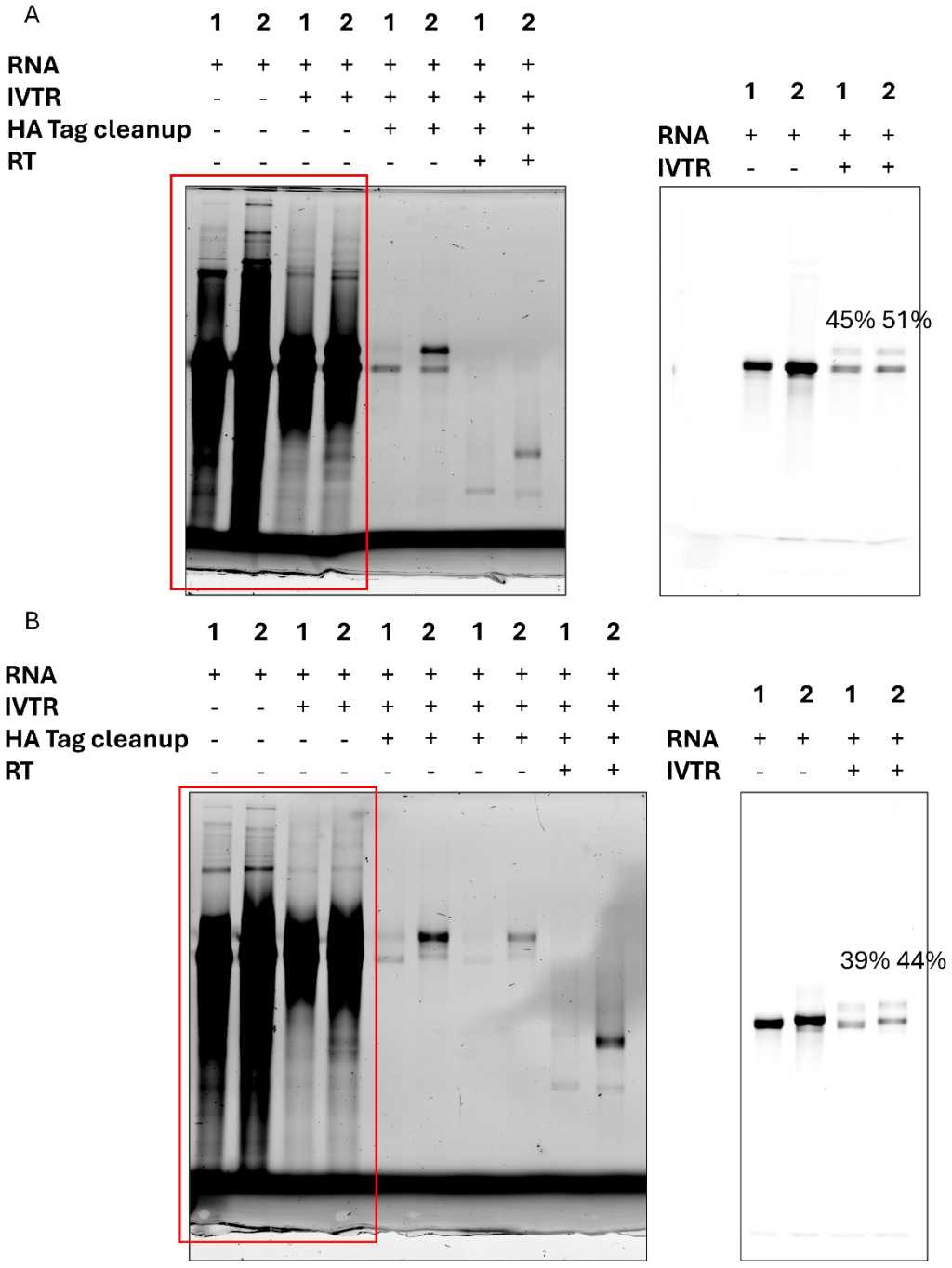
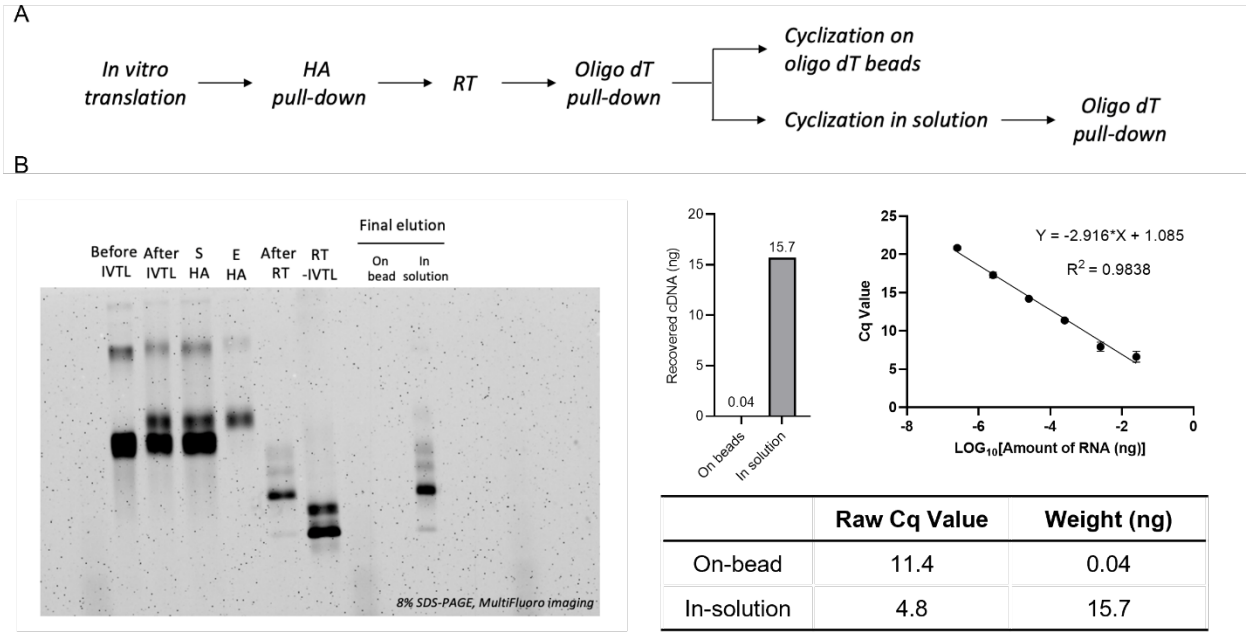
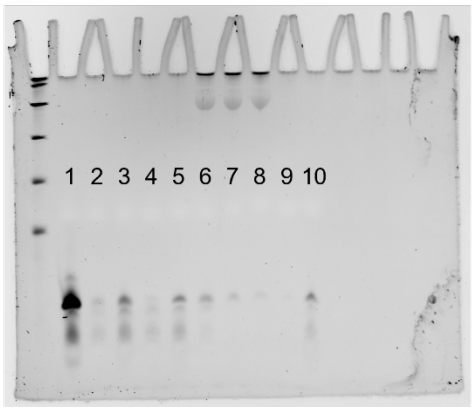


Figure S5. Gel validation of mRNA displayed peptide controls using 8% UREA-SDS-PAGE gel (120 V, 1.5 h). (**A**) Construct 1 and construct 2 correspond to ligated mRNA templates encoding peptide TBP-2 and TBP-1, respectively. Highlighted regions were adjusted to lower contrast and presented on the right for visualization of translated bands. A replicate experiment was performed, and results are shown in (**B**).

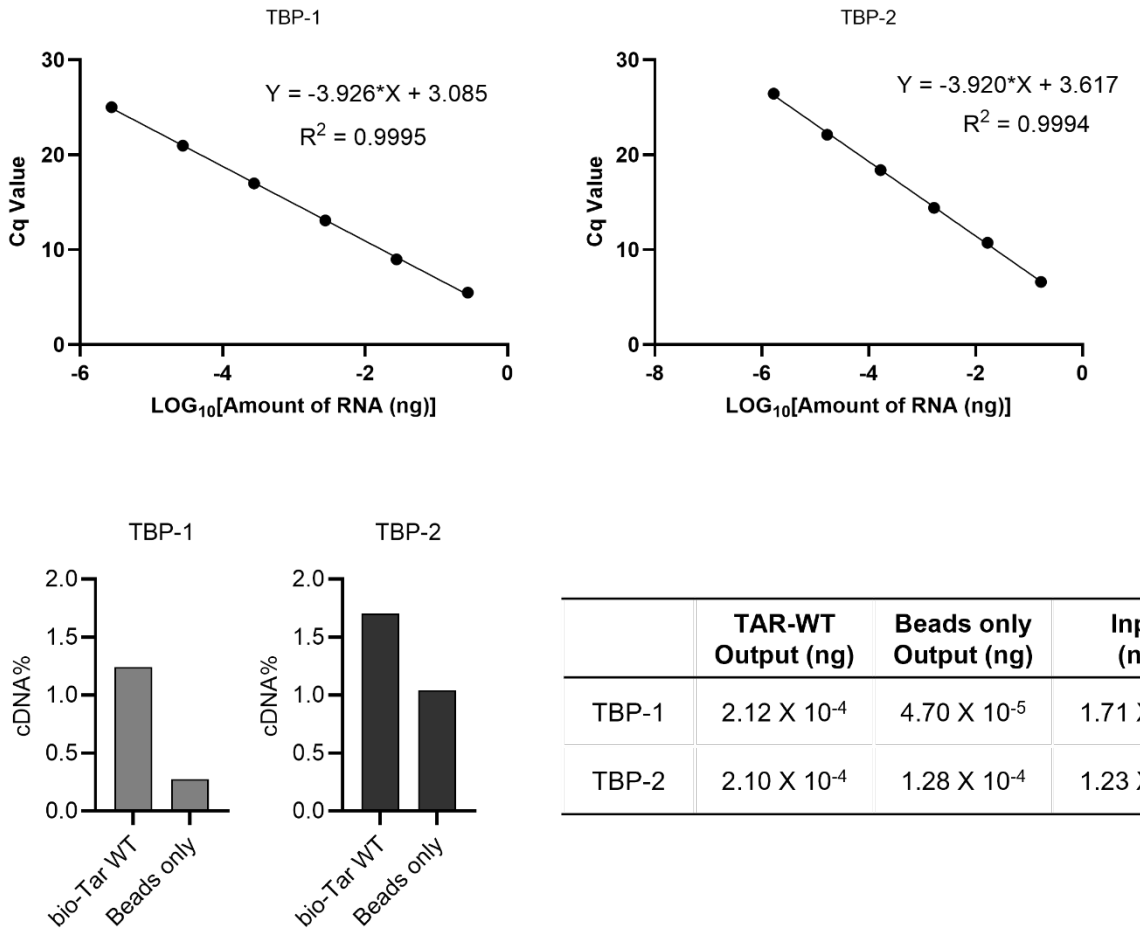


	Raw Cq Value	Weight (ng)
On-bead	11.4	0.04
In-solution	4.8	15.7



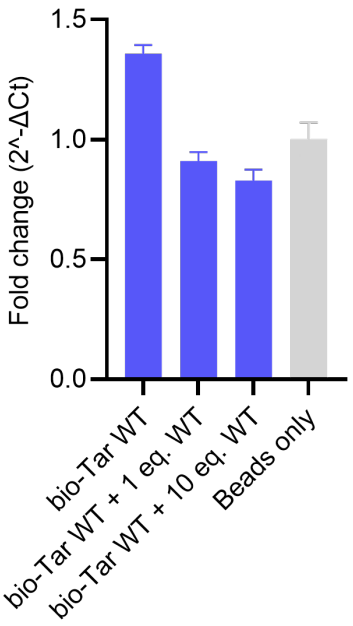
1 Folded 5'-bio HIV TAR WT
2 Supernatant (30 min)
3 Immobilized (30 min) eluted at 95 °C for 5 min
4 Supernatant (1h)
5 Immobilized (1h) eluted at 95 °C for 5 min
6 First wash (washing buffer)
7 Second wash (washing buffer)
8 Third wash (washing buffer)
9 Fourth wash (folding buffer)
10 Elution 95 °C for 5 min

Figure S6. Optimization of the mRNA display workflow. (**A**) Schematic representation of preparation of the mRNA display library. (**B**) Solution-phase cyclization yields a higher proportion of the cDNA-peptide complex compared to on-beads cyclization. (**C**) Optimization of washing conditions during the RNA immobilization step demonstrates that most RNA is immobilized on beads within 30 minutes, and additional washing steps does not remove bound RNA.



	TAR-WT Output (ng)	Beads only Output (ng)	Input (ng)
TBP-1	2.12×10^{-4}	4.70×10^{-5}	1.71×10^{-2}
TBP-2	2.10×10^{-4}	1.28×10^{-4}	1.23×10^{-2}

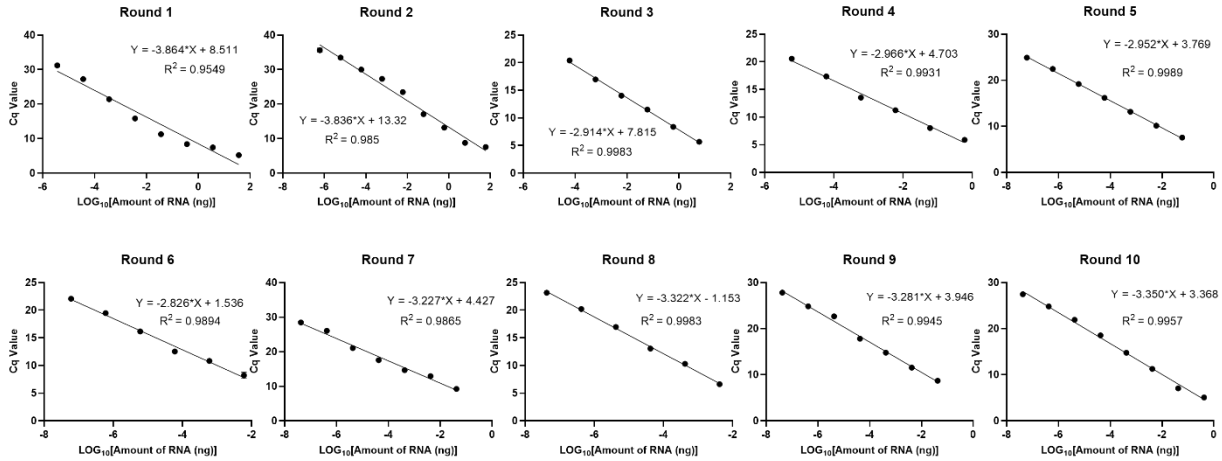
Figure S7. Quantitative PCR analysis of mRNA–TAR binding peptide conjugates (TBP-1 and TBP-2) captured in a streptavidin pull-down assay.



	Weight (ng)	Recovery (%)
Bio-Tar WT	3.68E-04	38.2
Bio-Tar WT + 1 eq. Tar WT	2.62E-04	27.2
Bio-Tar WT + 10 eq. Tar WT	2.42E-04	25.1
Beads only	2.84E-04	29.5
Input	9.63E-04	-

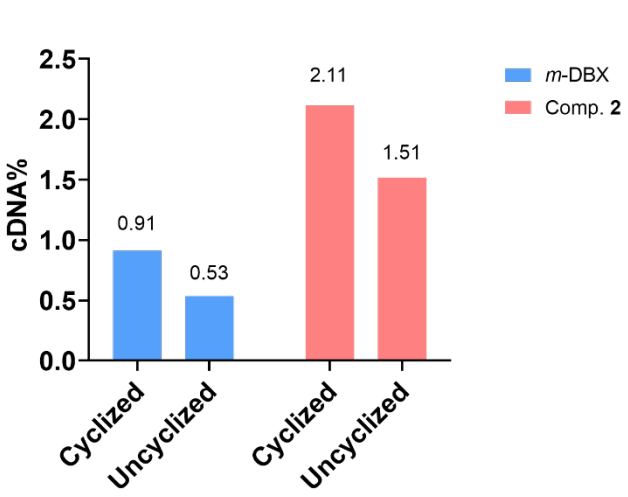
Figure S8. Competitive pull-down of TBP-1 displayed mRNA conjugate using excess unbiotinylated HIV TAR RNA.

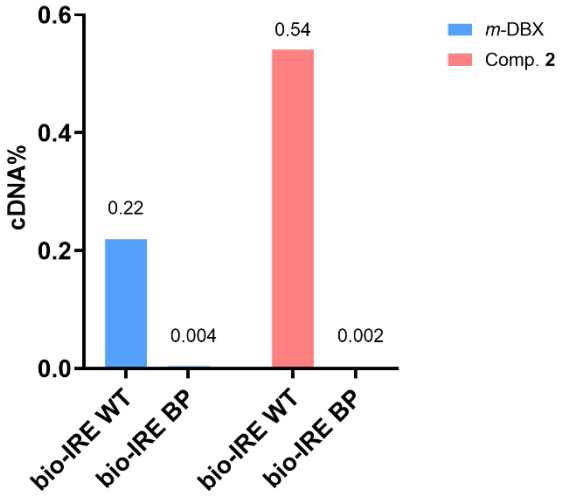
Additional qPCR and bioinformatic analysis of mRNA display selection outcomes for *SNCA* IRE (Figures S9-S14)



Round	*m*-DBX Output (ng)	*m*-DBX Input (ng)	*m*-DBX Recovery (%)	Comp. 2 Output (ng)	Comp. 2 Input (ng)	Comp. 2 Recovery (%)
1	2.8E-02	3.1E+00	9.1E-01	3.0E-02	2.6E+00	1.1E+00
2	1.6E-01	5.5E+01	2.8E-01	9.3E-02	4.7E+01	2.0E-01
3	1.6E-04	2.4E-01	6.8E-02	1.5E-04	1.7E-01	8.6E-02
4	1.6E-05	2.9E-02	5.4E-02	1.0E-05	2.7E-02	3.7E-02
5	8.5E-06	1.9E-02	4.5E-02	1.1E-05	1.3E-02	8.3E-02
6	1.8E-04	3.3E-01	5.6E-02	4.3E-04	4.4E-01	9.6E-02
7	5.0E-05	1.0E-01	4.8E-02	6.1E-05	4.8E-02	1.3E-01
8	4.7E-06	1.7E-03	2.7E-01	5.1E-06	7.0E-04	7.3E-01
9	1.3E-04	3.5E-02	3.7E-01	1.5E-04	3.5E-02	4.3E-01
10	3.5E-04	2.7E-02	1.3E+00	5.8E-04	5.8E-02	1.0E+00

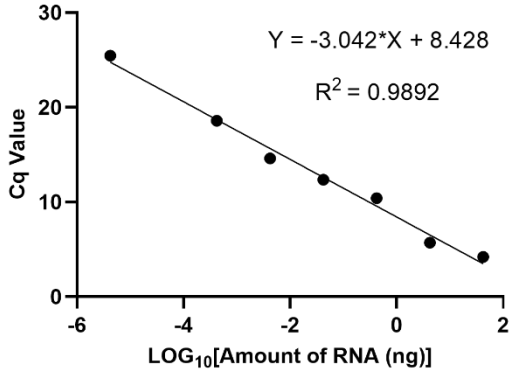
Figure S9. Standard curves for each round of selections and the standardized quantification of recovered cDNA.





	Cyclization	Weight (ng)
***m*-DBX**	Cyclized (Output)	2.6E-03
	Cyclized (Input)	2.9E-01
	Uncyclized (Output)	3.0E-03
	Uncyclized (Input)	5.6E-01
Comp. 2	Cyclized (Output)	5.0E-03
	Cyclized (Input)	2.4E-01
	Uncyclized (Output)	6.1E-03
	Uncyclized (Input)	4.0E-01

	Target	Weight (ng)
***m*-DBX**	Bio-IRE WT (Output)	2.4E-04
	Bio-IRE WT (Input)	1.1E-01
	Bio-IRE BP (Output)	7.2E-06
	Bio-IRE BP (Input)	1.7E-01
Comp. 2	Bio-IRE WT (Output)	1.7E-03
	Bio-IRE WT (Input)	3.1E-01
	Bio-IRE BP (Output)	7.6E-06
	Bio-IRE BP (Input)	3.7E-01



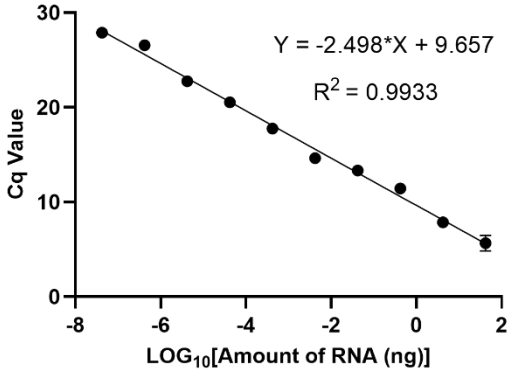


Figure S10. qPCR validation of the recovered cDNA library after 10 rounds of selection against *SNCA* IRE.



	Selection round	Total sequence	Barcode retrieved	Passed Quality filter	Number of cysteines			Length between 2 cysteines				
					1-cysteine	2-cysteine	0 and multiple cysteines	6	8	10	12	Others
***m*-DBX library**	**5**	360217	345202	265600	3239	236282	26079	60734	48996	96667	28933	952
	6	213533	201163	154168	2733	132071	19364	28358	22717	60325	20054	617
	7	328132	278385	235301	6331	189817	39153	32124	26826	100386	29400	1081
	8	279818	231197	194826	6986	148417	39423	17033	16521	90034	23567	1262
	9	304900	263448	208997	7674	158040	43283	9761	11728	113500	21371	1680
	10	248844	233640	150337	0	129044	21293	1854	4583	110286	12033	288
Comp 2. library	**5**	297686	283488	220767	1494	143757	75516	28750	25820	64964	23312	911
	6	285961	271043	214727	740	114556	99431	8546	13546	67756	23810	898
	7	292272	245093	213296	552	125407	87337	2509	11432	88692	22030	744
	8	307981	249632	214544	728	137036	76780	1376	15983	100280	18619	778
	9	266545	224071	192768	749	132519	59500	551	17561	99345	14520	542
	10	329407	314332	226718	6	169120	57592	434	20491	123642	24374	179

Figure S11. Overview of the sequencing data analysis workflow and the corresponding number of sequences retained at each processing step.

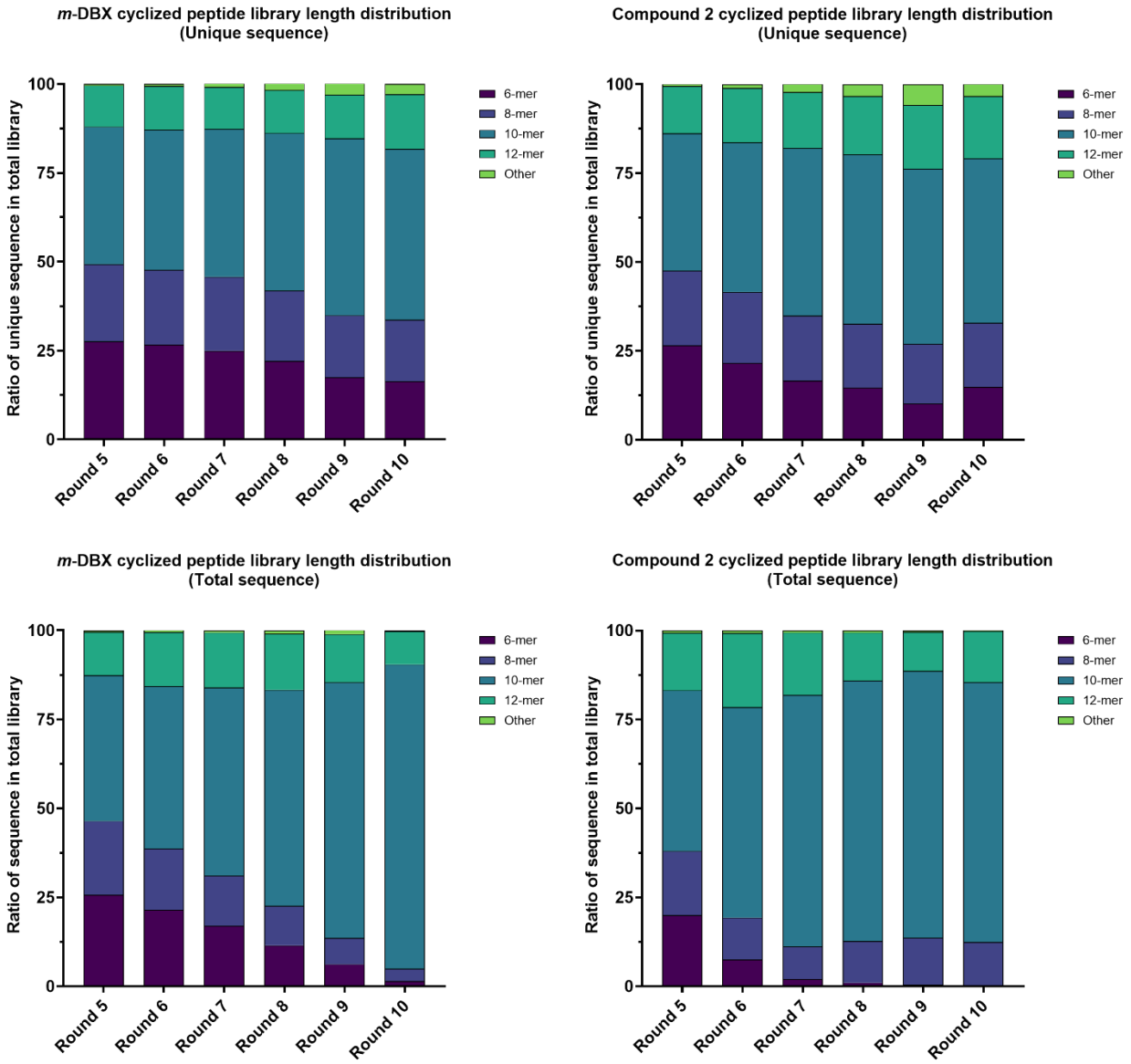


Figure S12. The length distribution analysis of the di-cysteine peptides from selection rounds 5-10 reveals an increasing proportion of 10-mer and 12-mer variants, while the percentage of 6-mer and 8-mer variants declines.

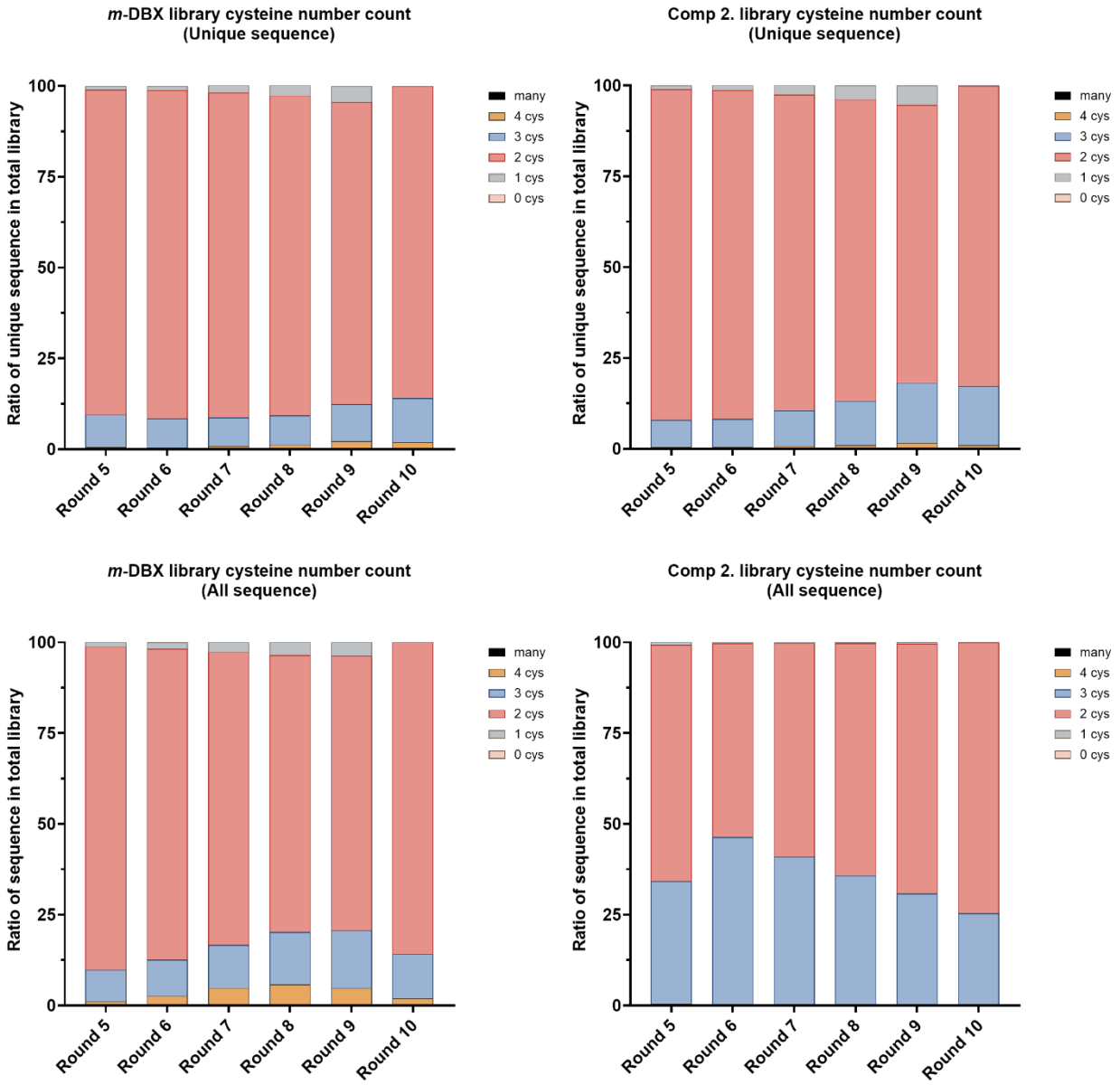
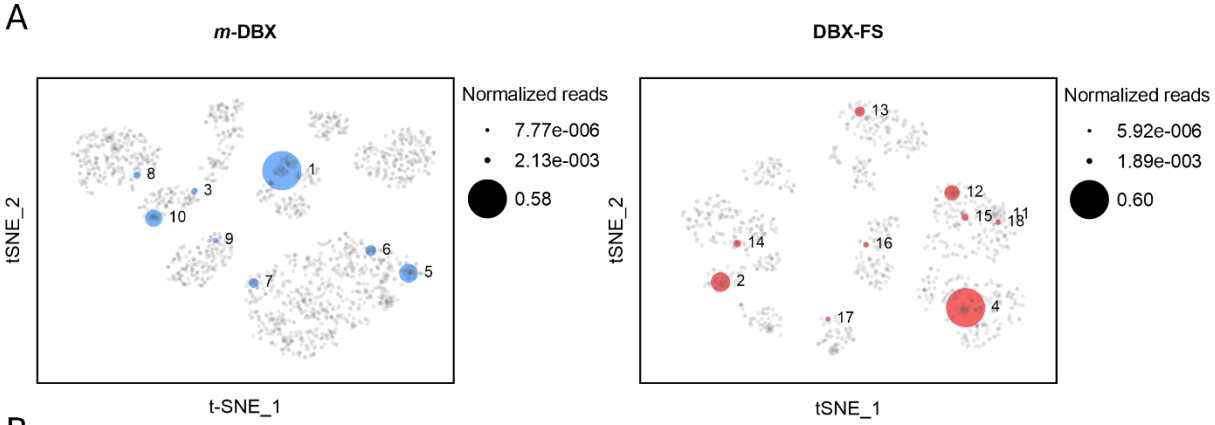


Figure S13. Cysteine composition analysis from selection rounds 5-10 reveals that the majority of variants contain two cysteines, with minimal mutations observed.

A



B

Peptide sequence	Name	%Reads
MCPPTTTRWQPHC	1	58.6
MCRYKTTSMIC	3	0.25
MCSTTDAPAYAAC	5	10.4
MCTTTTNPLYC	6	2.2
MCSTTTVKDLPPC	7	1.6
MCKTTTSVC	8	0.05
MCDTTRVRDPAEYPC	9	0.1
MCGQTTVDDLRNLLC	10	8.3
MCSTTYDFRRTTRFC	2	11.7
MCSTTNMLPNLSC	4	60.5
MCPYSTTFDPC	11	11.9
MCSTTLVLYPQSC	12	6.3
MCITTTNPHPLSC	13	1.7
MCLNSTTTDANKC	14	0.6
MCYTTTTNTLPLC	15	0.5
MCRPTTSVFTFEGMC	16	0.1
MCDRTTSLIPLEKWC	17	0.04
MCSTTFDPC	18	0.1

Figure S14. t-distributed Stochastic Neighbor Embedding (t-SNE) analysis of all unique peptides after ten rounds of selection. (**A**) t-SNE plots for both libraries, with representative peptides from major clusters highlighted. (**B**) Selected peptides from each library and their corresponding percentage reads.

Binding affinity measurements for *SNCA* IRE hit peptides (Figure S15-S18)

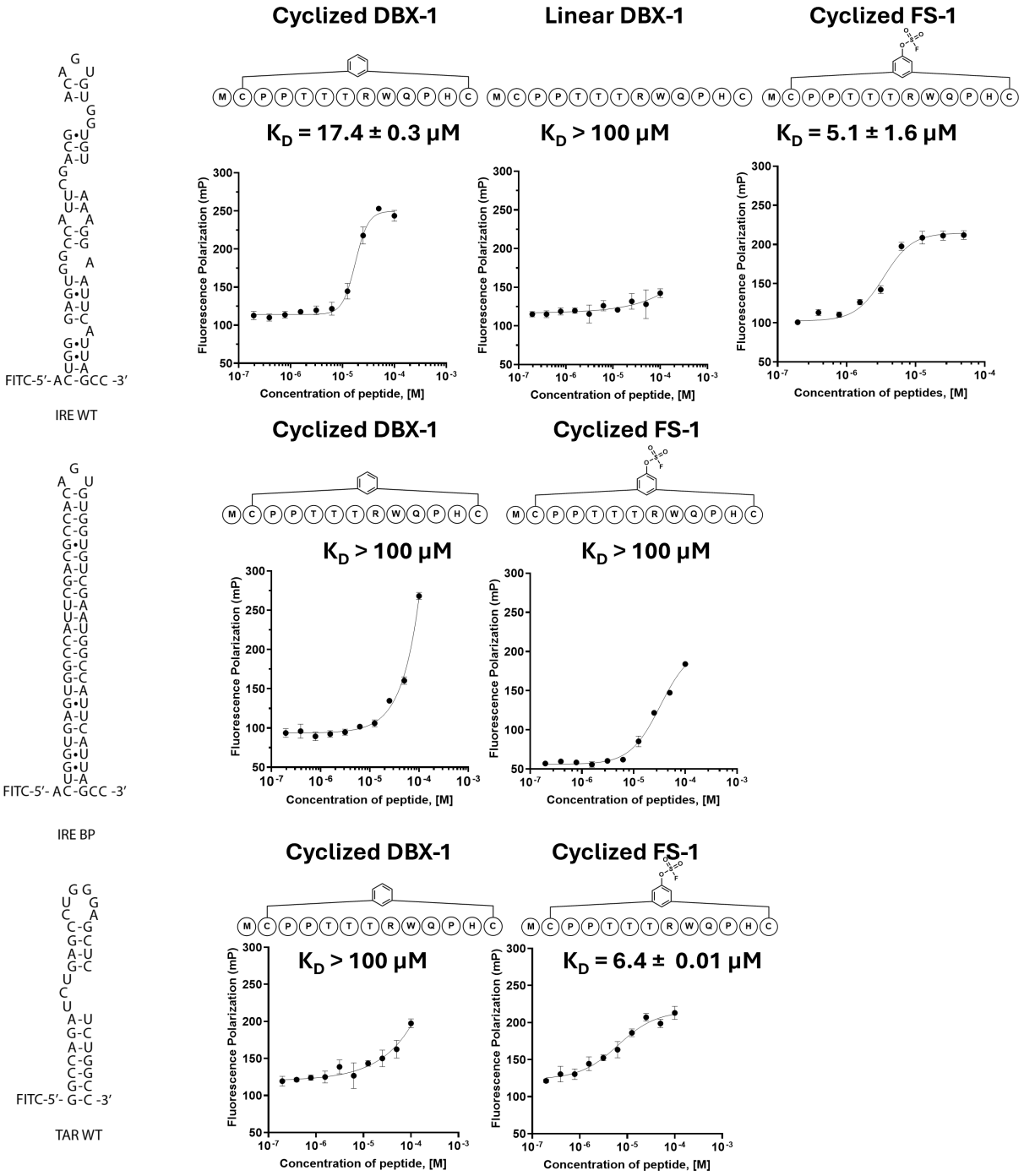


Figure S15. *In vitro* binding characterization of cyclized DBX-1 and FS-1 to the *SNCA* IRE, a fully base-paired control and HIV TAR determined by fluorescence polarization assay.

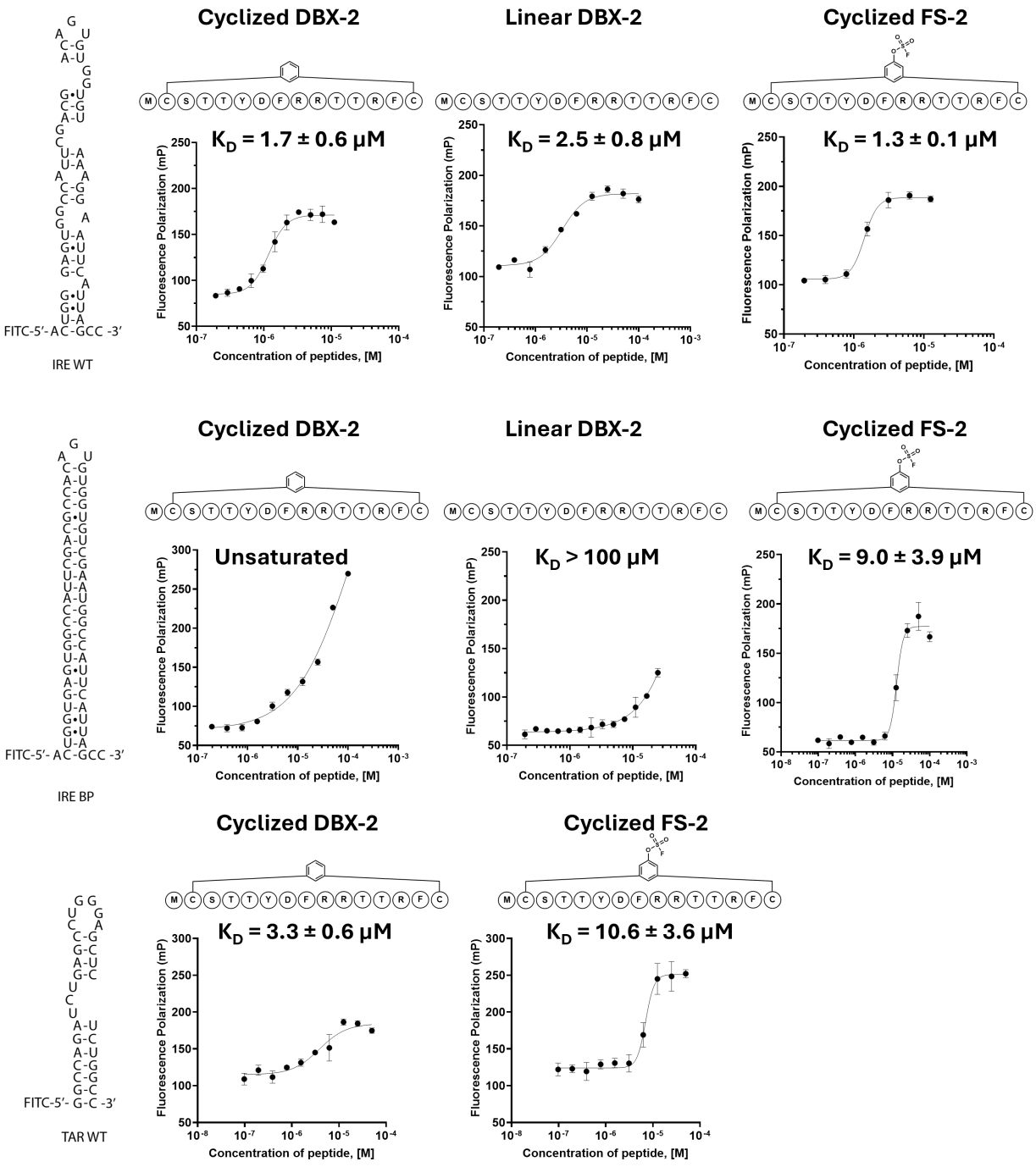


Figure S16. *In vitro* binding characterization of cyclized DBX-2 and FS-2 to the *SNCA* IRE, a fully base-paired control and HIV TAR determined by fluorescence polarization assay.

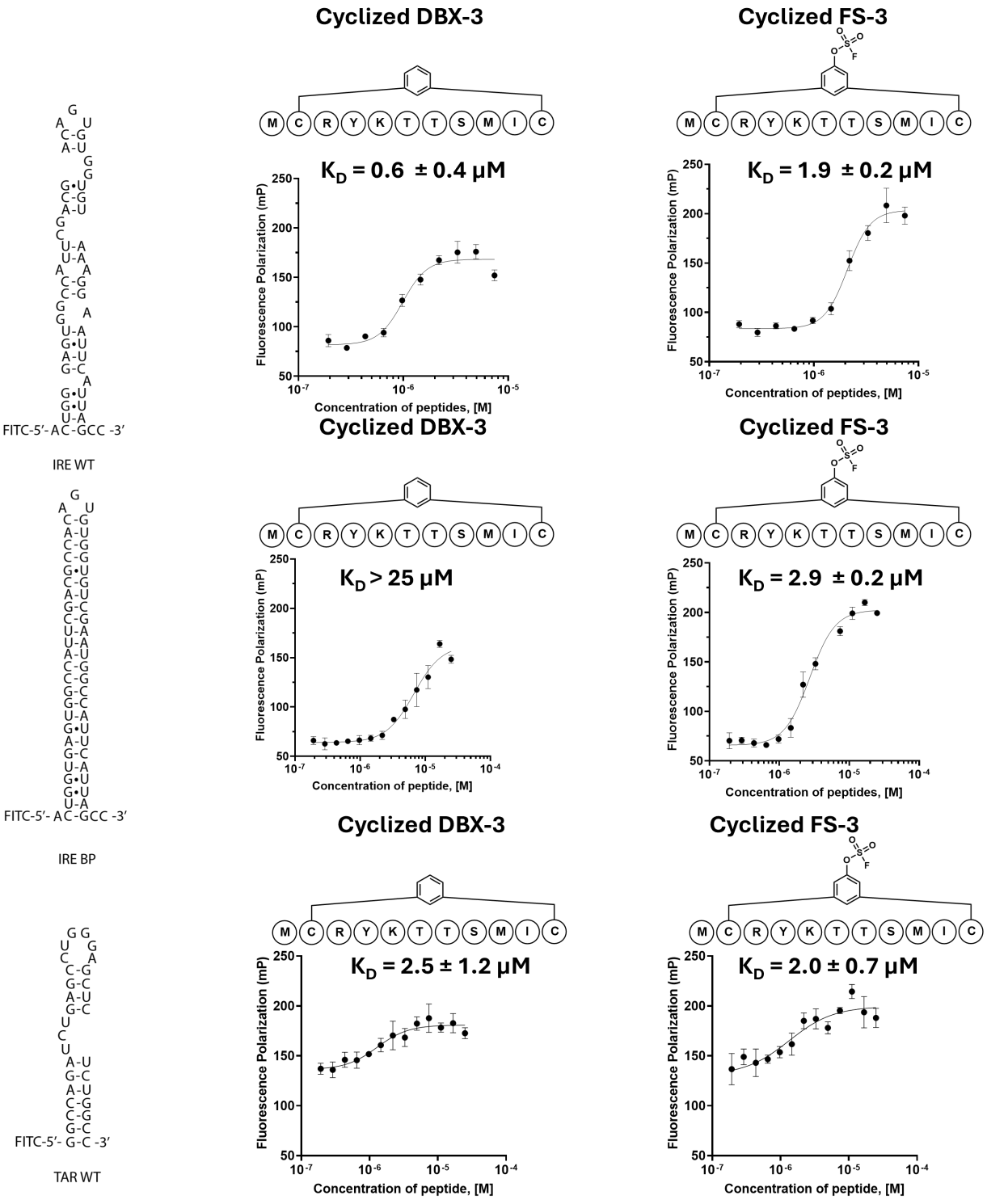


Figure S17. *In vitro* binding characterization of cyclized DBX-3 and FS-3 to the *SNCA* IRE, a fully base-paired control and HIV TAR as determined by fluorescence polarization assay.

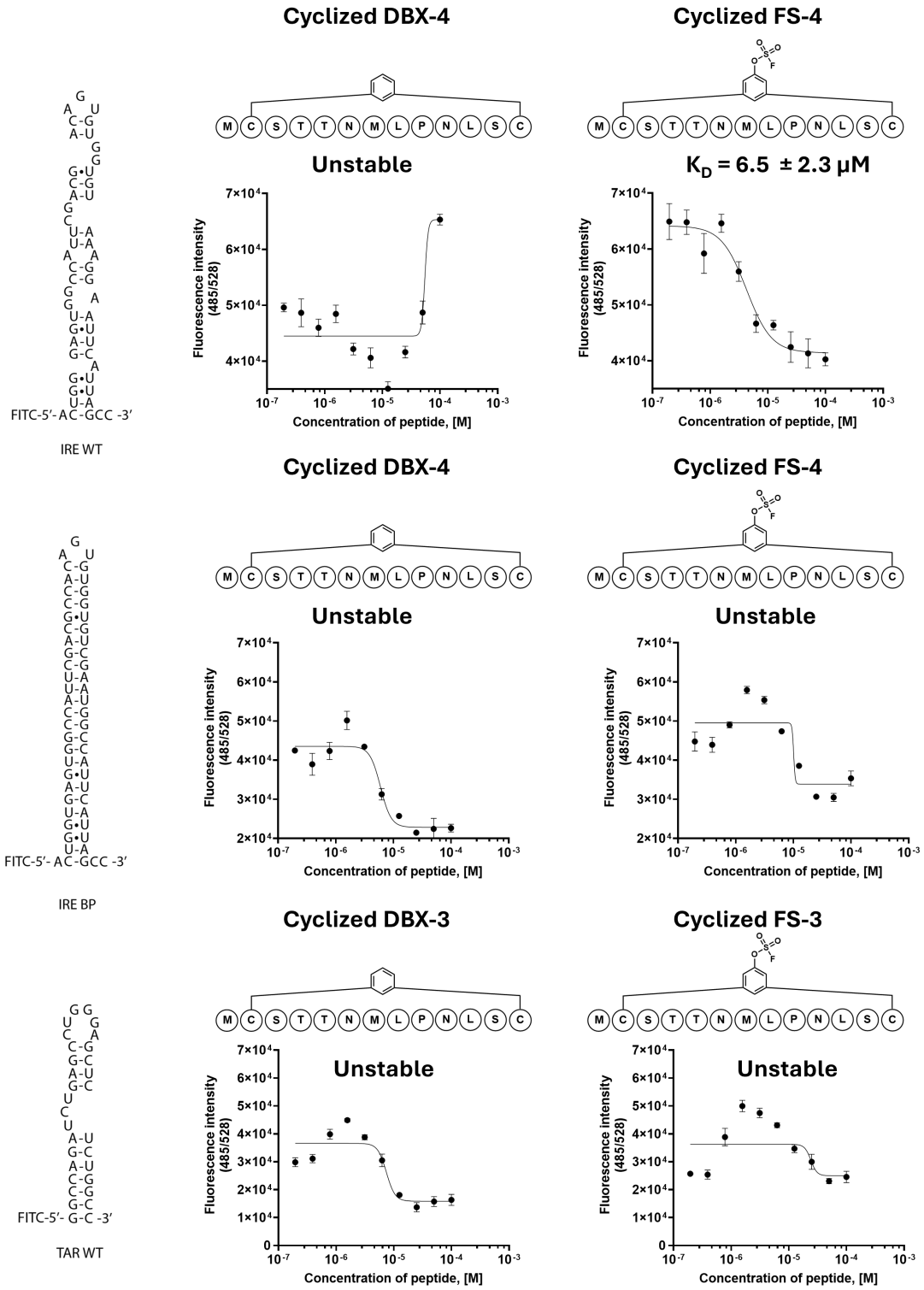


Figure S18. *In vitro* binding characterization of cyclized DBX-4 and FS-4 to the *SNCA* IRE, a fully base-paired control and HIV TAR determined by fluorescence quenching assay. No binding observed by fluorescence polarization assay using DBX-4 or FS-4 for these three targets.

Amino acid enrichment changes across six rounds of selection (Figure S19)

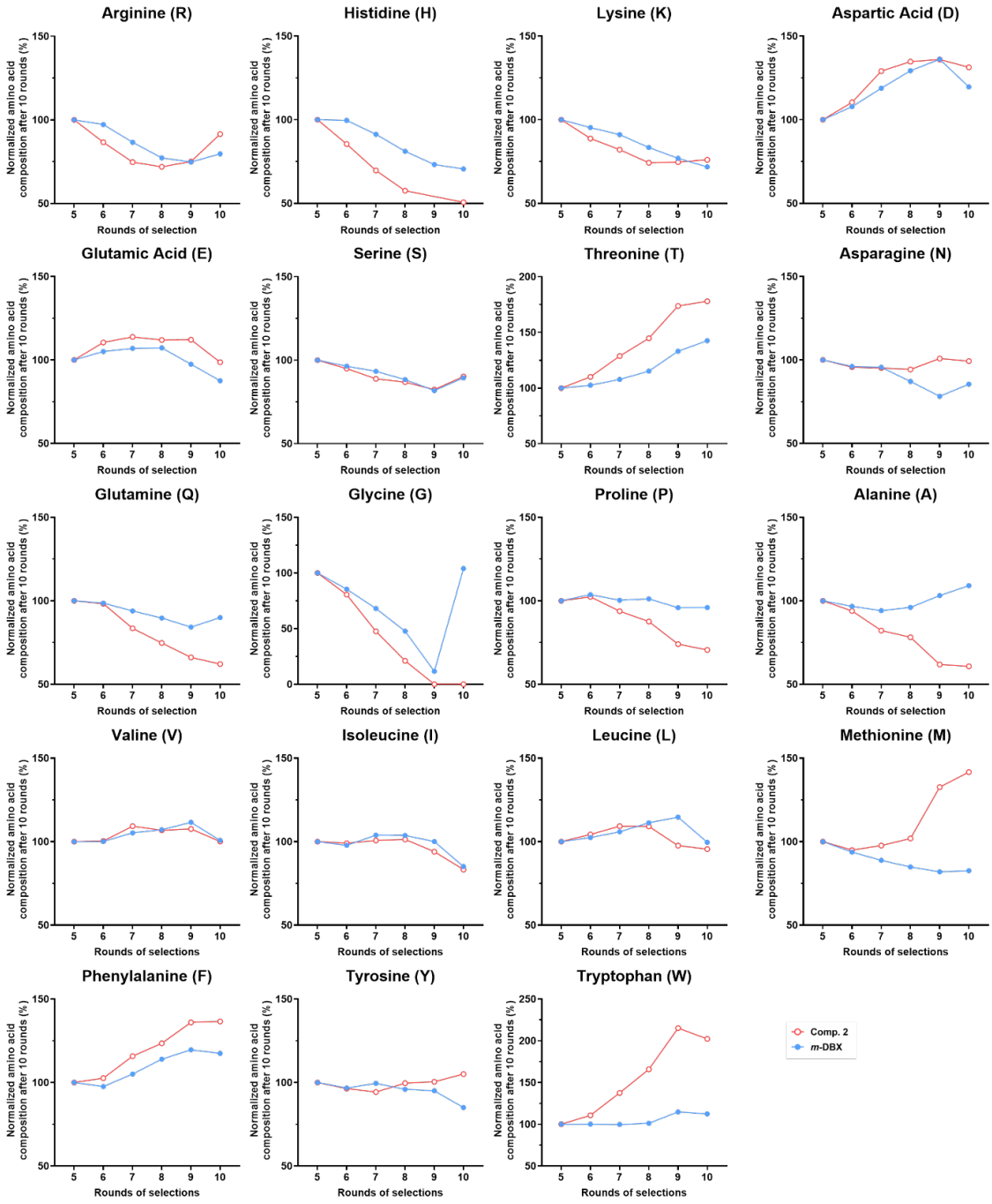


Figure S19. Enrichment of amino acids from rounds 5-10. The ratios of each amino acid (except cysteine) were calculated and normalized to translation bias for NNK degenerate codon followed by normalization to round 5. The trend records this ratio change after round 5.

Electrophoretic Mobility Shift Assay (EMSA) for detecting covalent peptides-RNA interactions (Figure S20-S23)

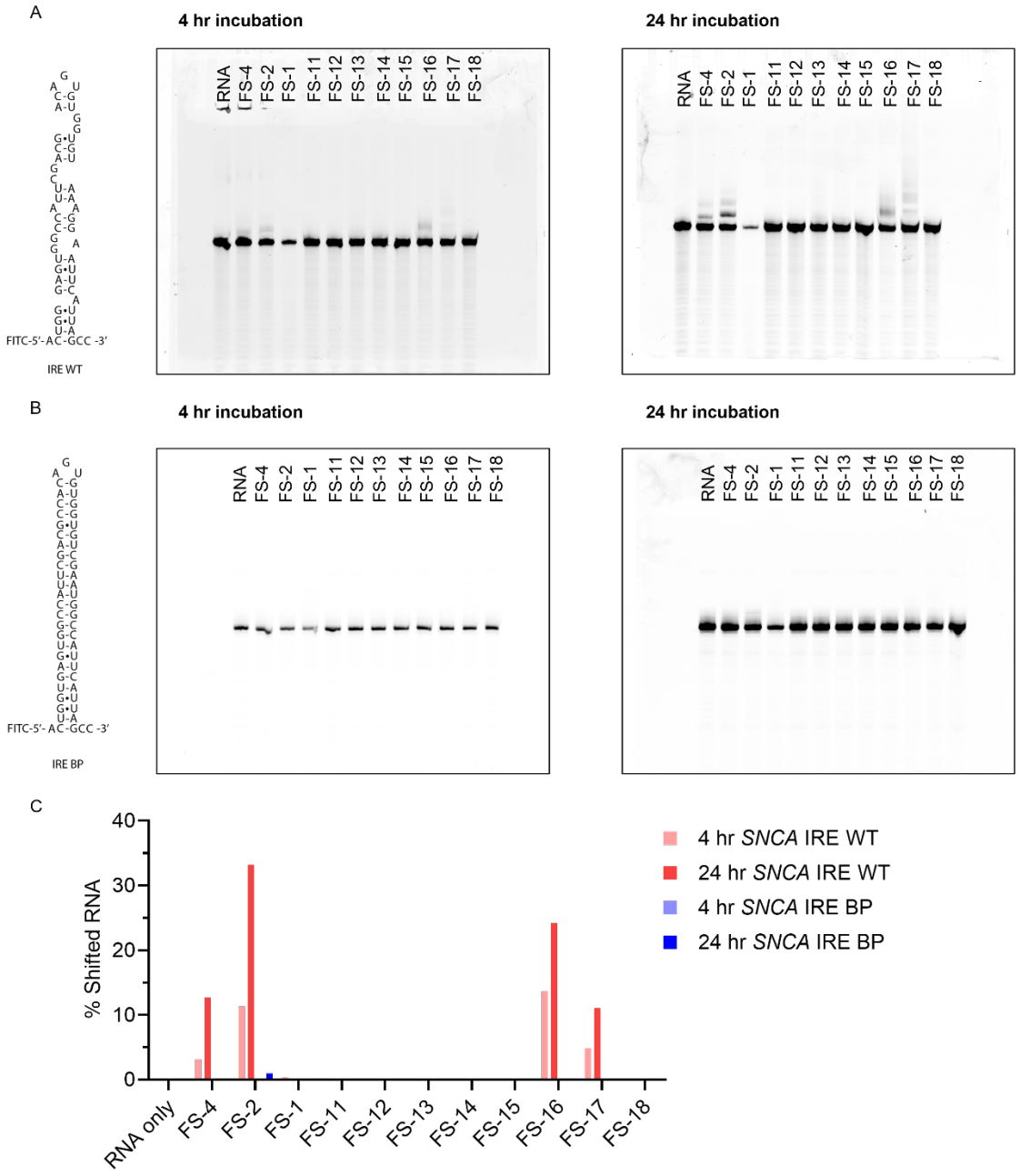


Figure S20. Denaturing 15% PAGE gel mobility shift assay following incubation of peptide hits with *SNCA* IRE or base-paired control. (Peptide FS-1, FS-2, FS-4 were purified from solid phase synthesized peptides. All other peptides were obtained from custom synthesis of the linear peptide followed by purification. Then cyclization was achieved with compound **2** without further purification. Desired cyclized products were confirmed by MALDI-TOF as major products. 1 μM RNA were incubated with 100 μM (Peptide to RNA, 100:1) compound at room temperature in 20 mM HEPES buffer (1 mM $MgCl_2$, pH 7.5).

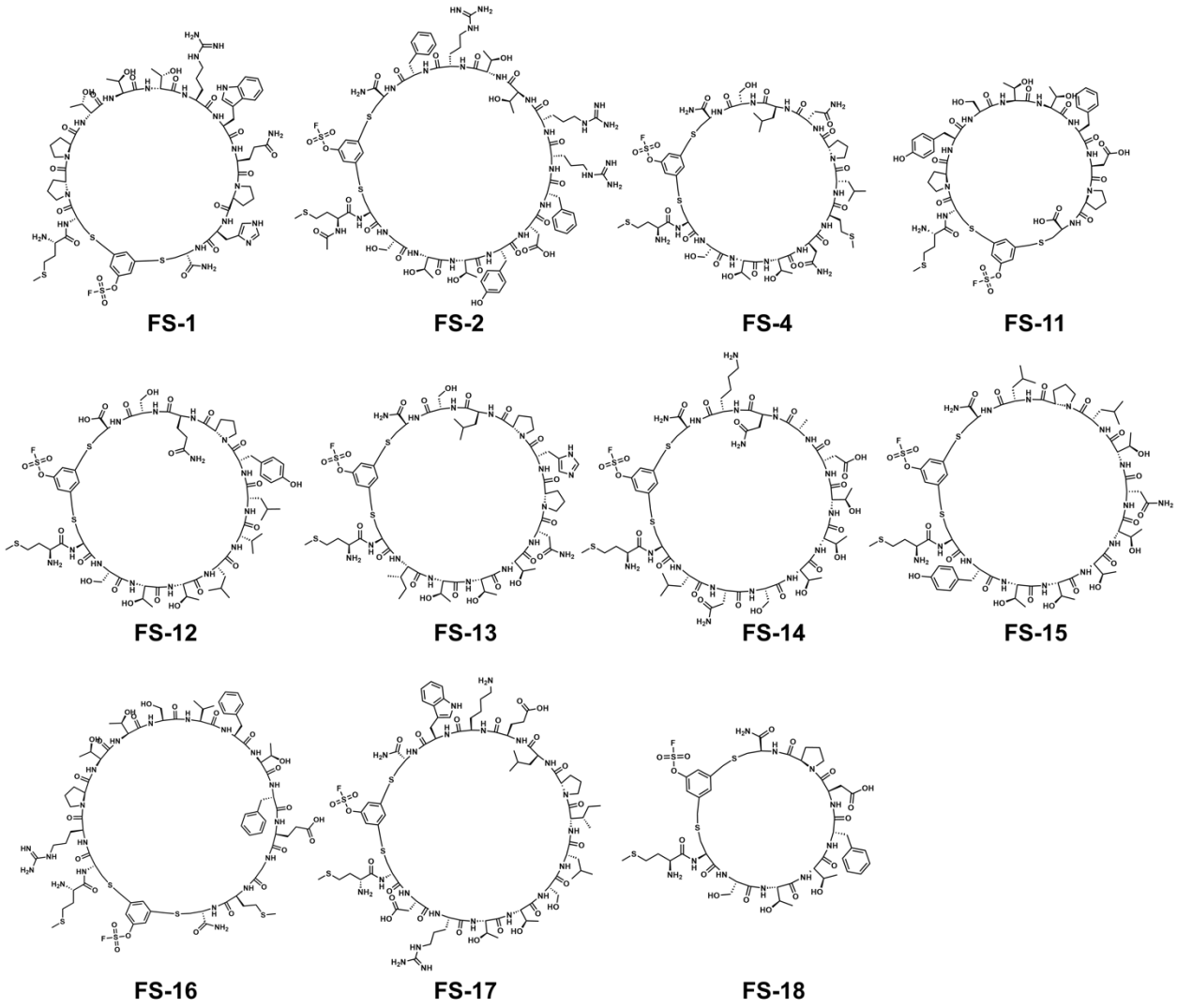


Figure S21. Structures of covalent peptide hits screened in denaturing gel shift assay.

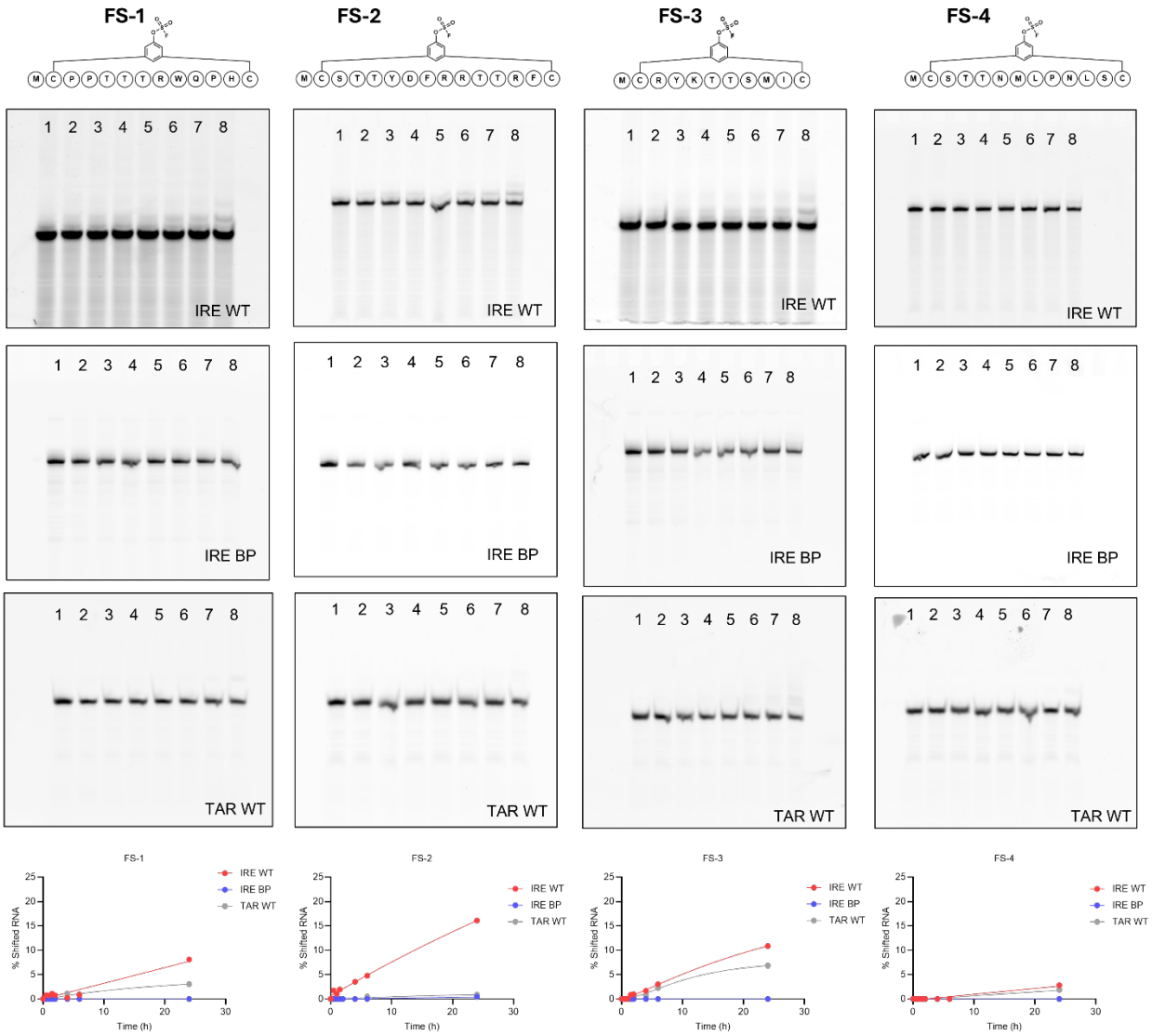


Figure S22. Four covalent peptide hits shows time dependent RNA labeling by denaturing PAGE gel mobility shift assay following incubation of with SNCA IRE, a fully base-paired IRE control, and HIV TAR. Lane 1 represents the untreated control (no peptide), while lanes 2 to 8 correspond to incubation times of 0.5, 1, 1.5, 2, 4, 6, and 24 hours. 1 μM RNA were incubated with 50 μM (Peptide to RNA, 50:1) compound at room temperature.

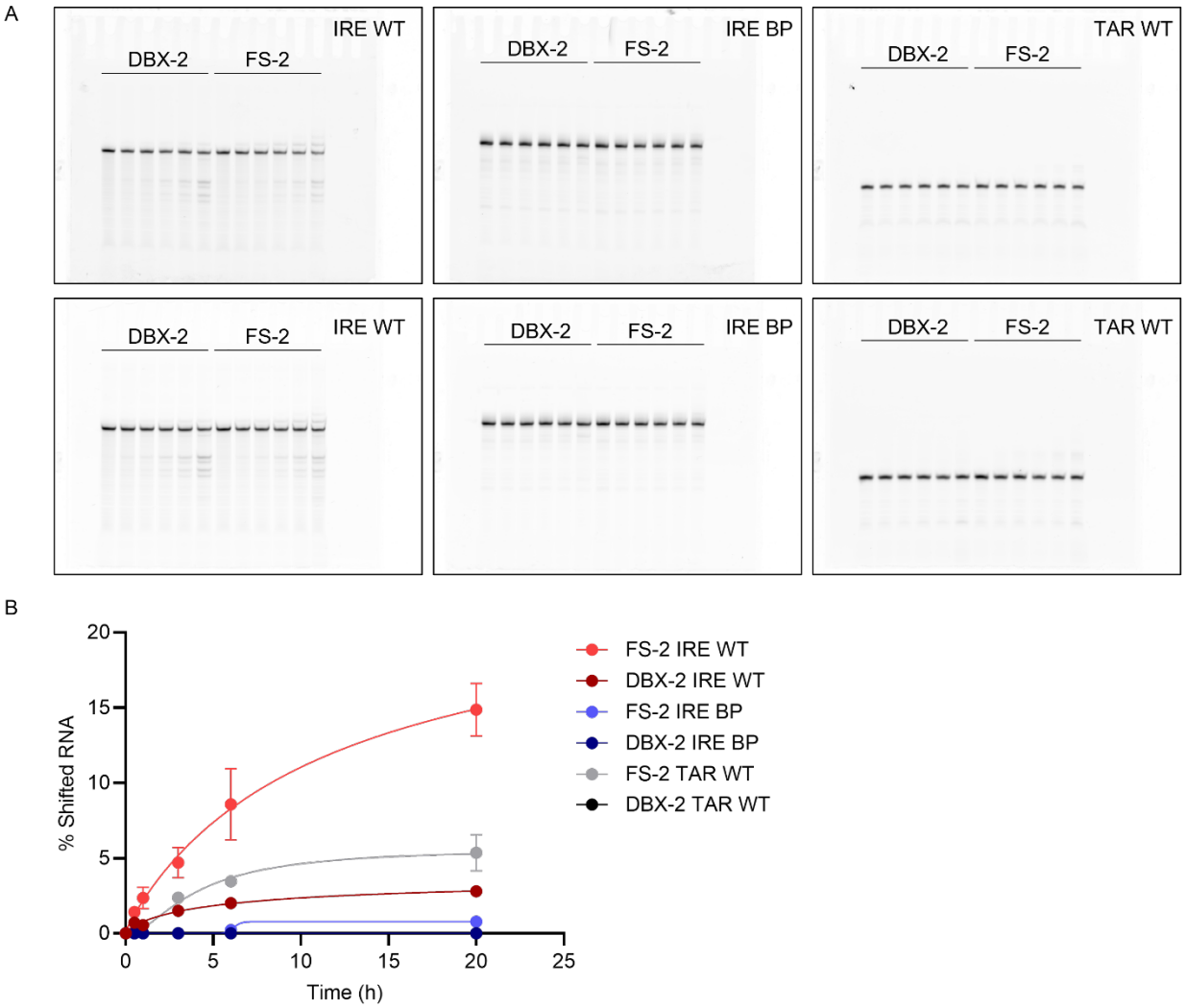


Figure S23. Denaturing 15% PAGE gel mobility shift assay following incubation of peptide hits with *SNCA* IRE, a fully base-paired IRE control, and HIV TAR. Lanes correspond to incubation times of 0, 0.5, 1, 3, 6, and 20 hours (left to right). 0.5 μM RNA were incubated with 50 μM (Peptide to RNA, 100:1) compound at 37 °C. Error bars represent SEM from two independent experiments.

Rate constant measurement for RNA labeling by FS-2 (Figure S24-S26)

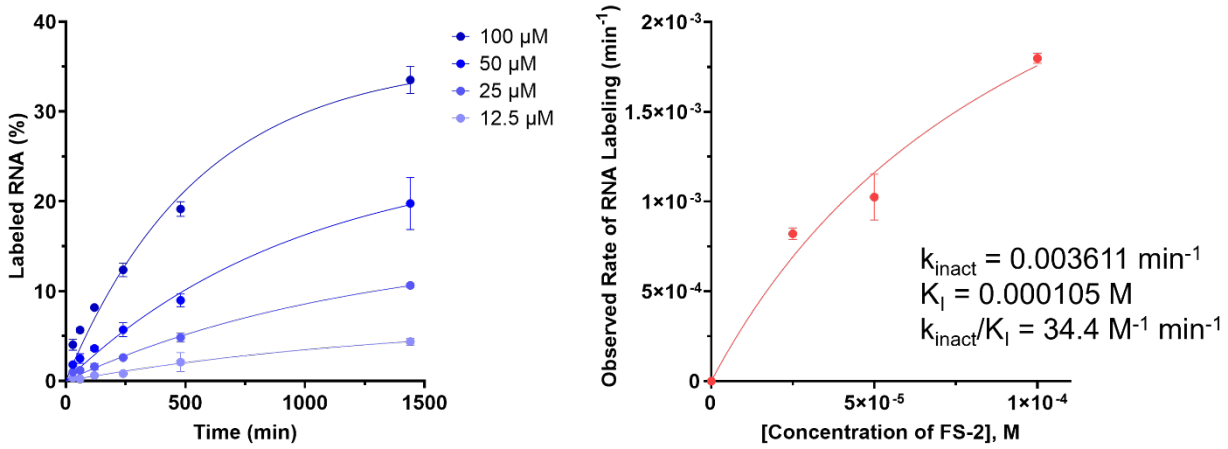


Figure S24. Concentration dependent RNA labeling by FS-2. Left, time course analysis of RNA labeling at a final concentration of 2.5 μM with varying concentrations of FS-2. Experiments were performed in 20 mM HEPES, pH 7.5, 1 mM $MgCl_2$ at 25 °C. Right, the plot of observed rate of RNA labeling as a function of FS-2 concentration. The observed rates were determined by quantifying the shifted RNA bands from denaturing gel electrophoresis. Error bars represent the standard error of the mean from two independent experiments.

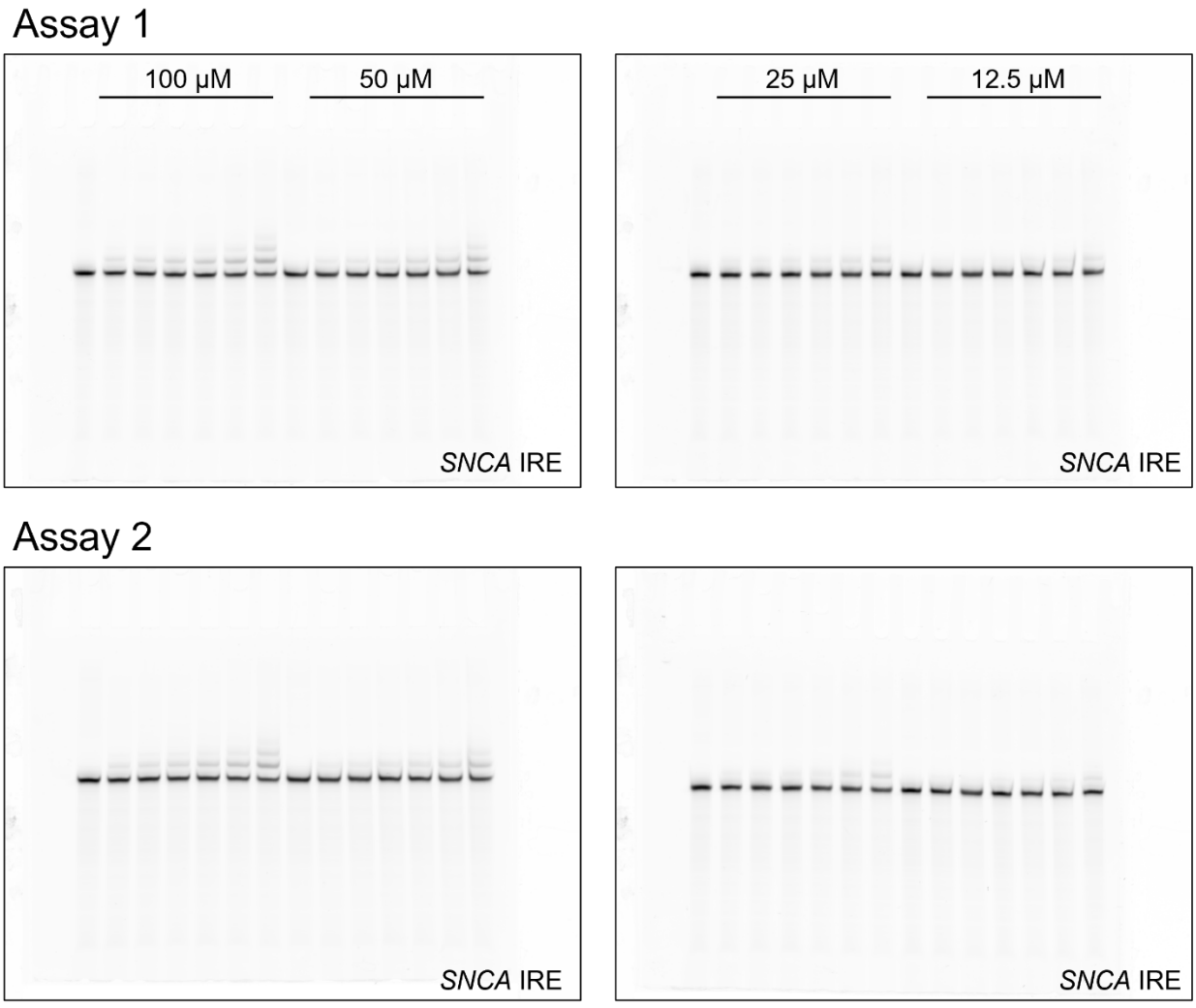


Figure S25. Complete gel images show time course of RNA labeling by FS-2. Lanes from left to right represent time points 0, 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h. Four different concentrations of FS-2 were used in each assay.

Effect of ionic strength and BSA additive on RNA labeling by FS-2 (Figure S26)

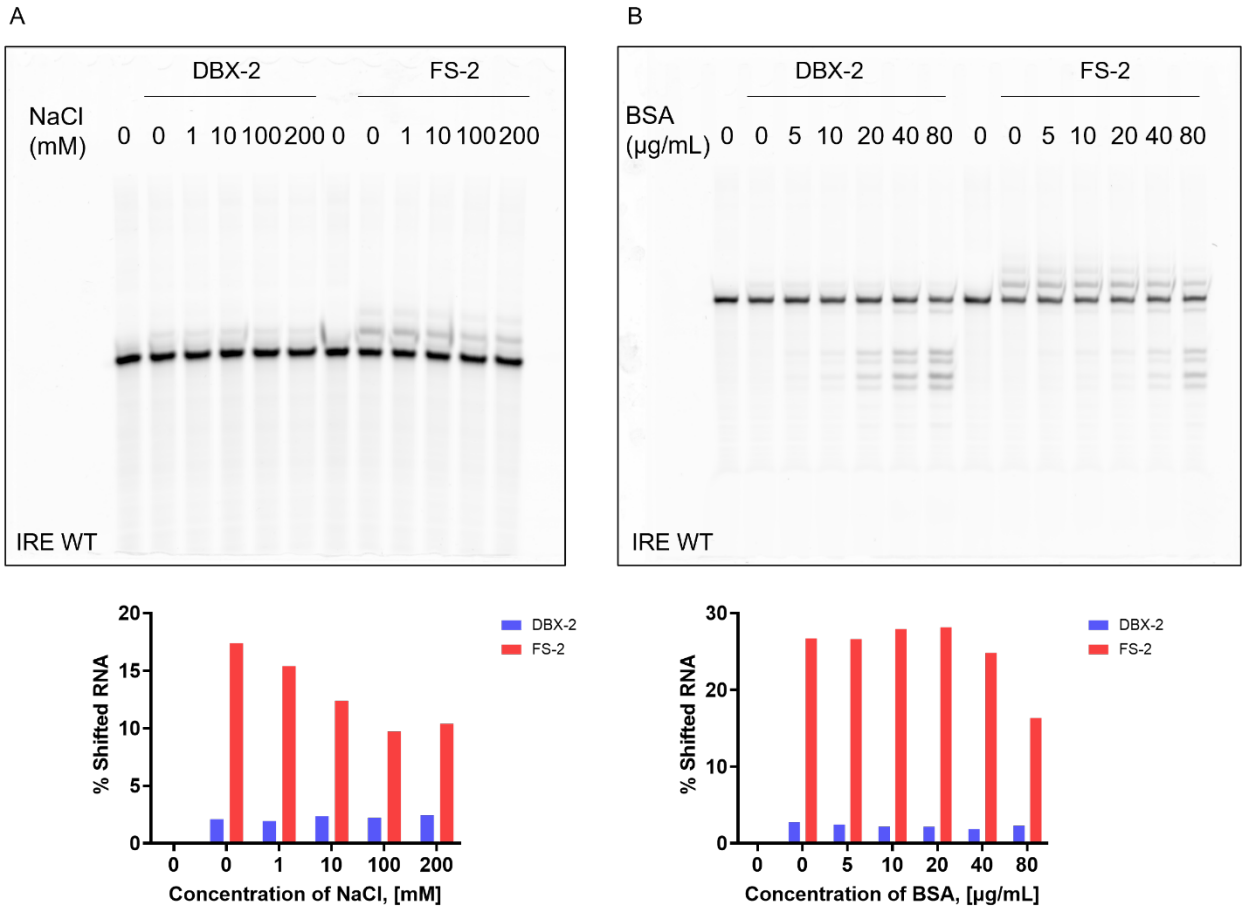


Figure S26. Effect of ionic strength and BSA additive on RNA labeling by FS-2. Reactions were incubated at room temperature for 24 hours in 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$. 1 µM RNA were incubated with 50 µM FS-2. A) Increasing concentrations of NaCl resulted in reduced RNA labeling by FS-2. B) The addition of BSA showed minimum impact on labeling efficiency. (Higher concentrations of BSA led to visible RNA degradation, likely due to RNase contamination).

FS-2 warhead stability and reactivity assessment (Figure S27-S32)

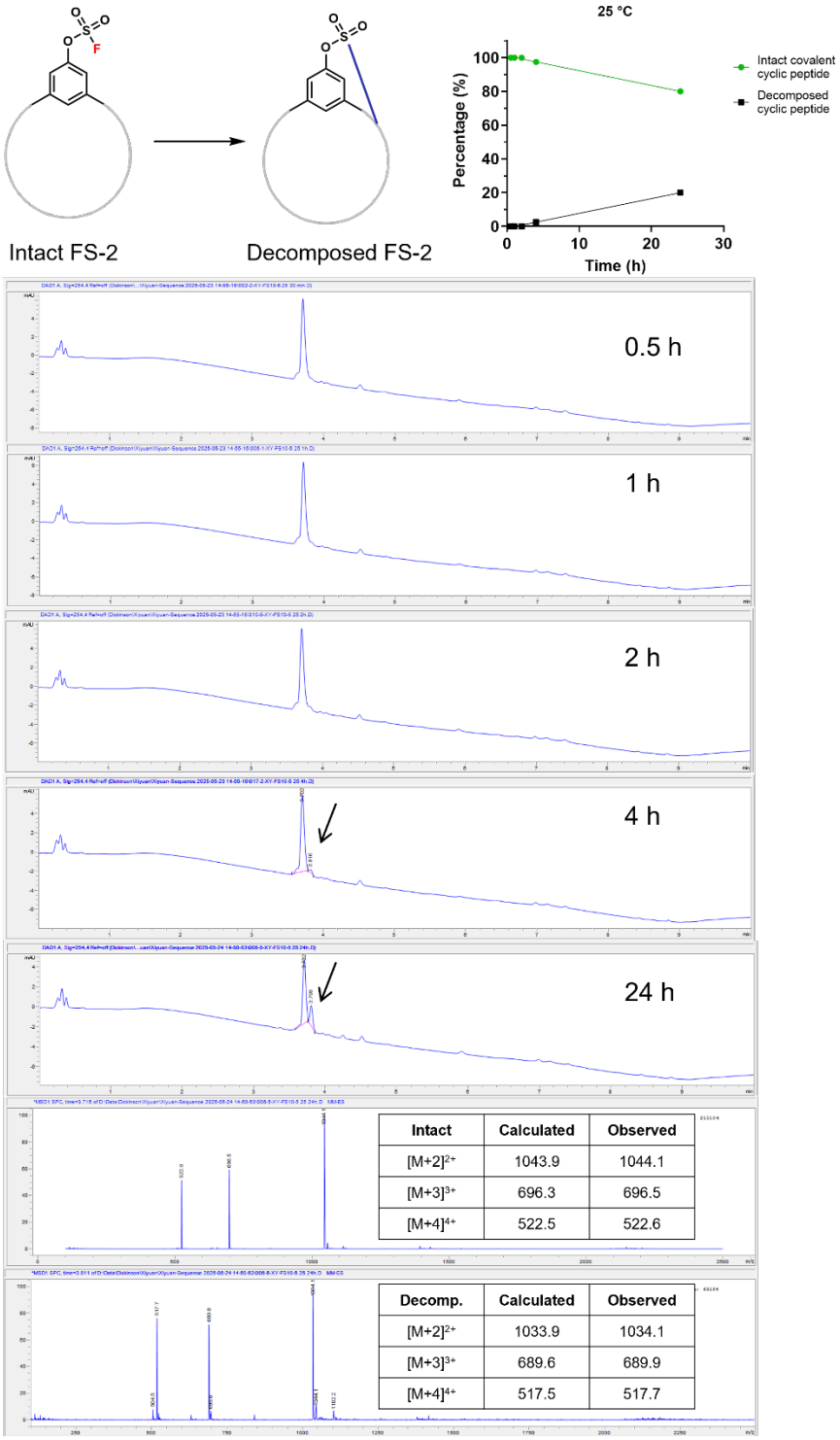


Figure S27. Stability test of FS-2 at 25 °C in HEPES buffer (pH 7.5).

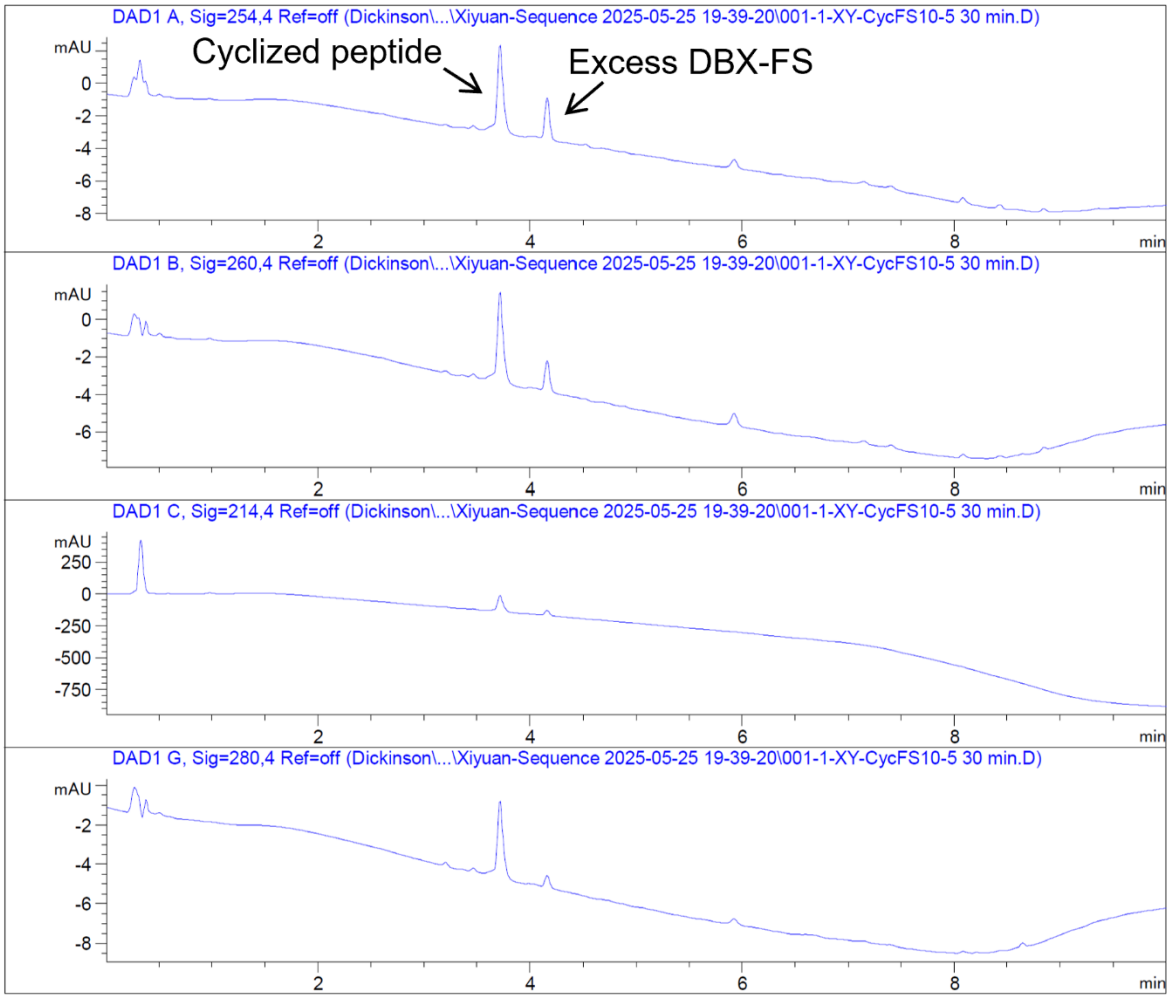


Figure S28. Cyclization of the linear peptide (FS-2) was monitored by ESI-LC-MS and demonstrated rapid conversion to the cyclic form within 30 minutes, with minimal detectable linear starting material or side products.

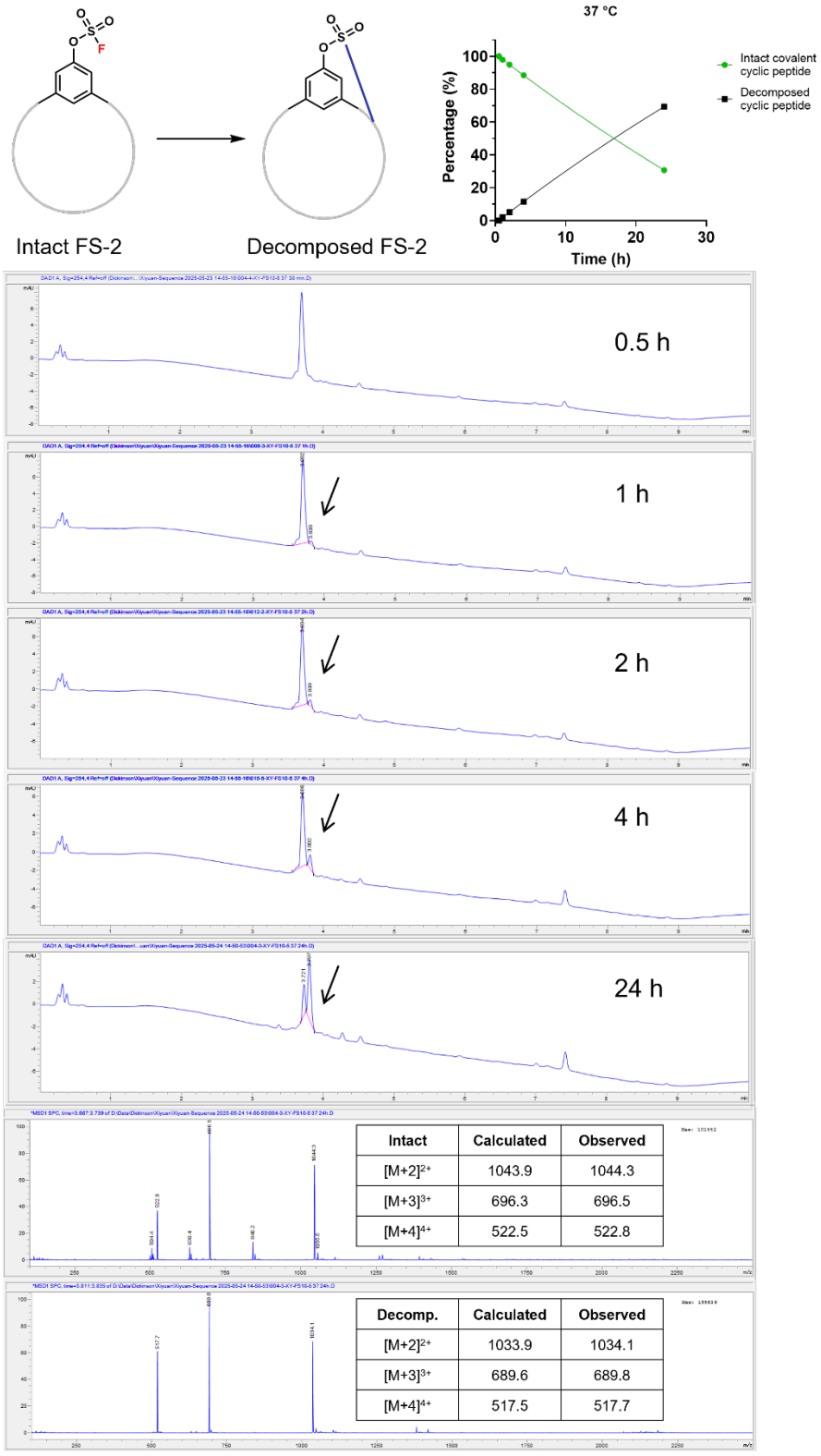


Figure S29. Stability test of FS-2 at 37 °C in HEPES buffer (pH 7.5).

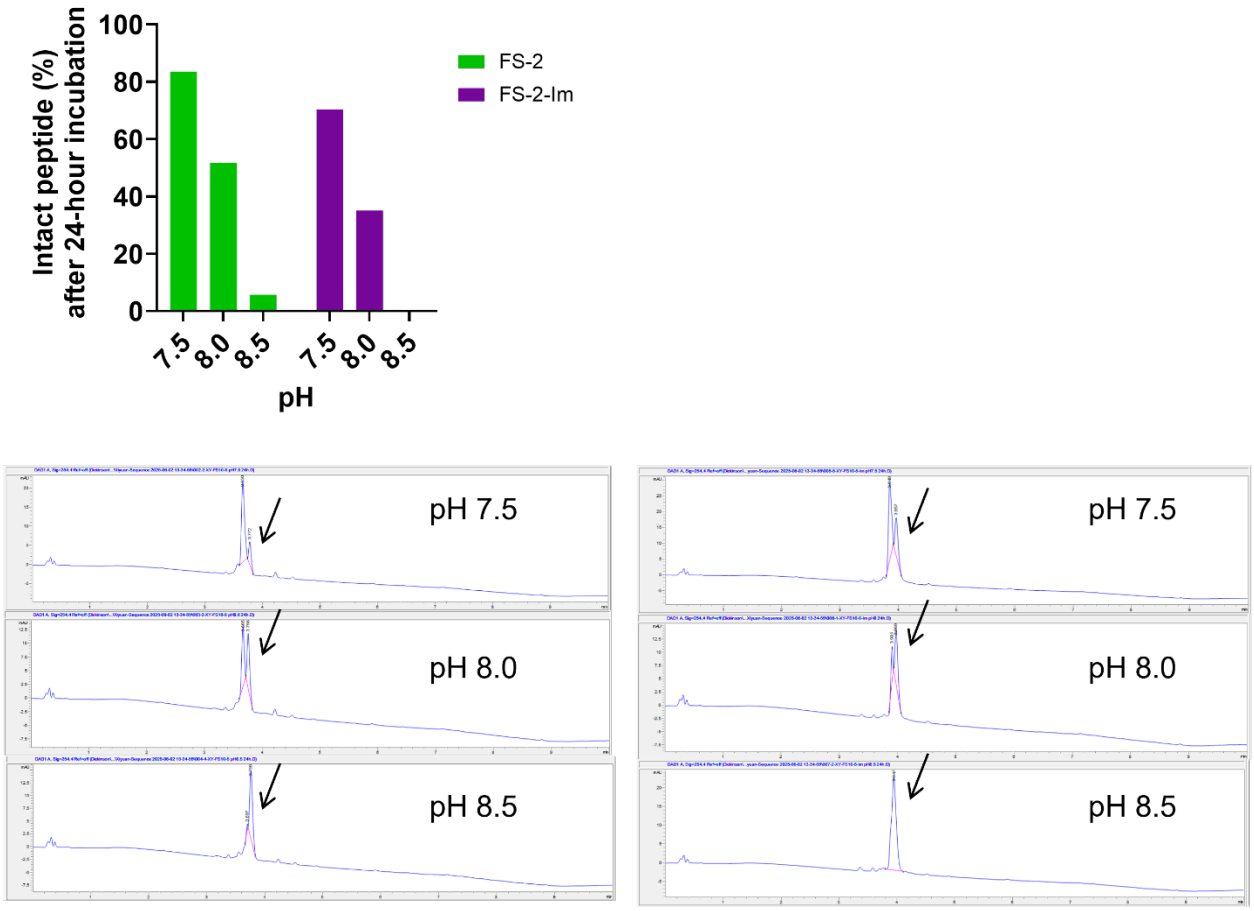


Figure S30. Stability assessment of FS-2 and FS-2-Im at 25 °C in HEPES buffer at different pH.

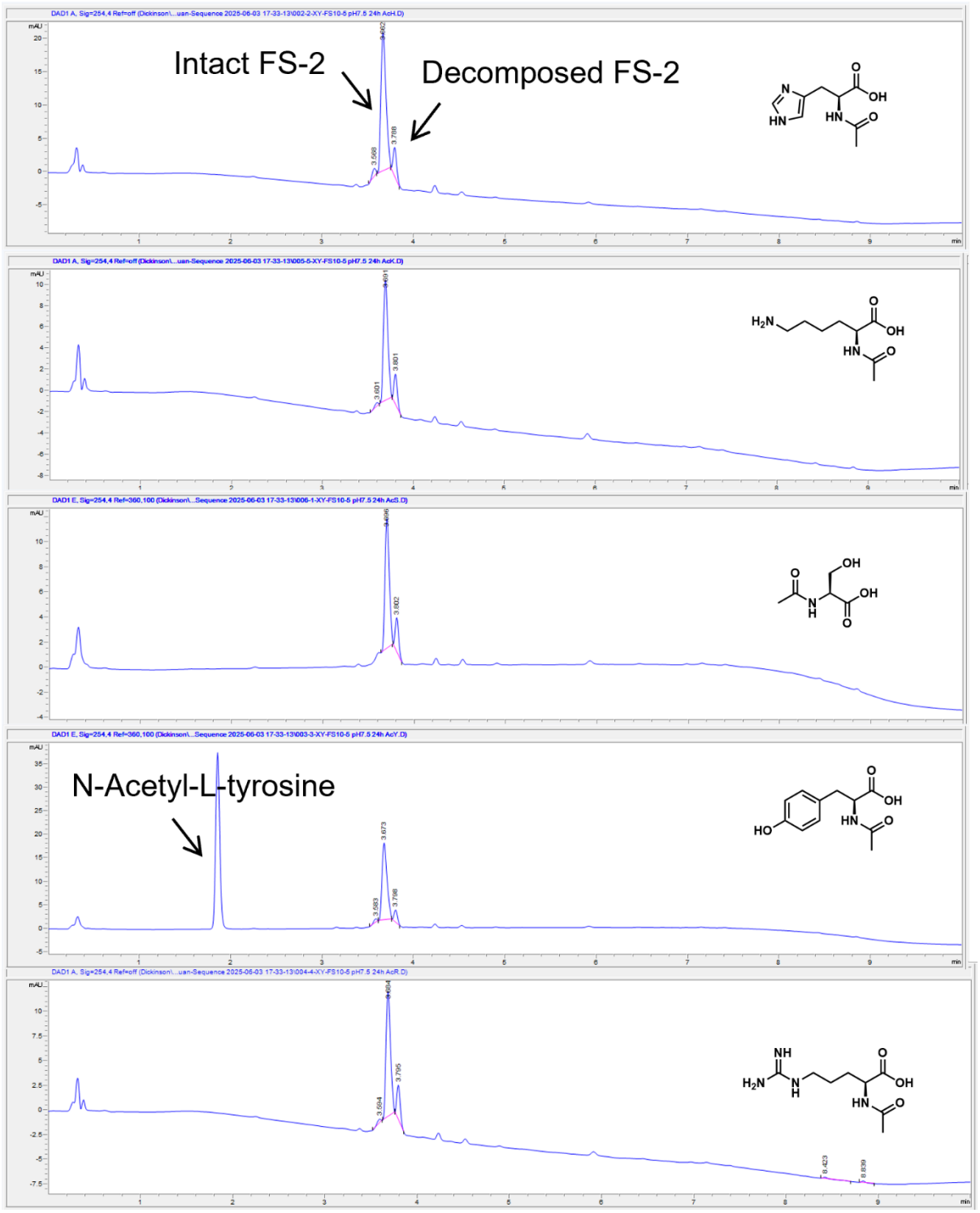


Figure S31. FS-2 exhibits minimal reactivity toward five N-acetylated amino acids (2 mM) after overnight incubation at pH 7.5, 25 °C.

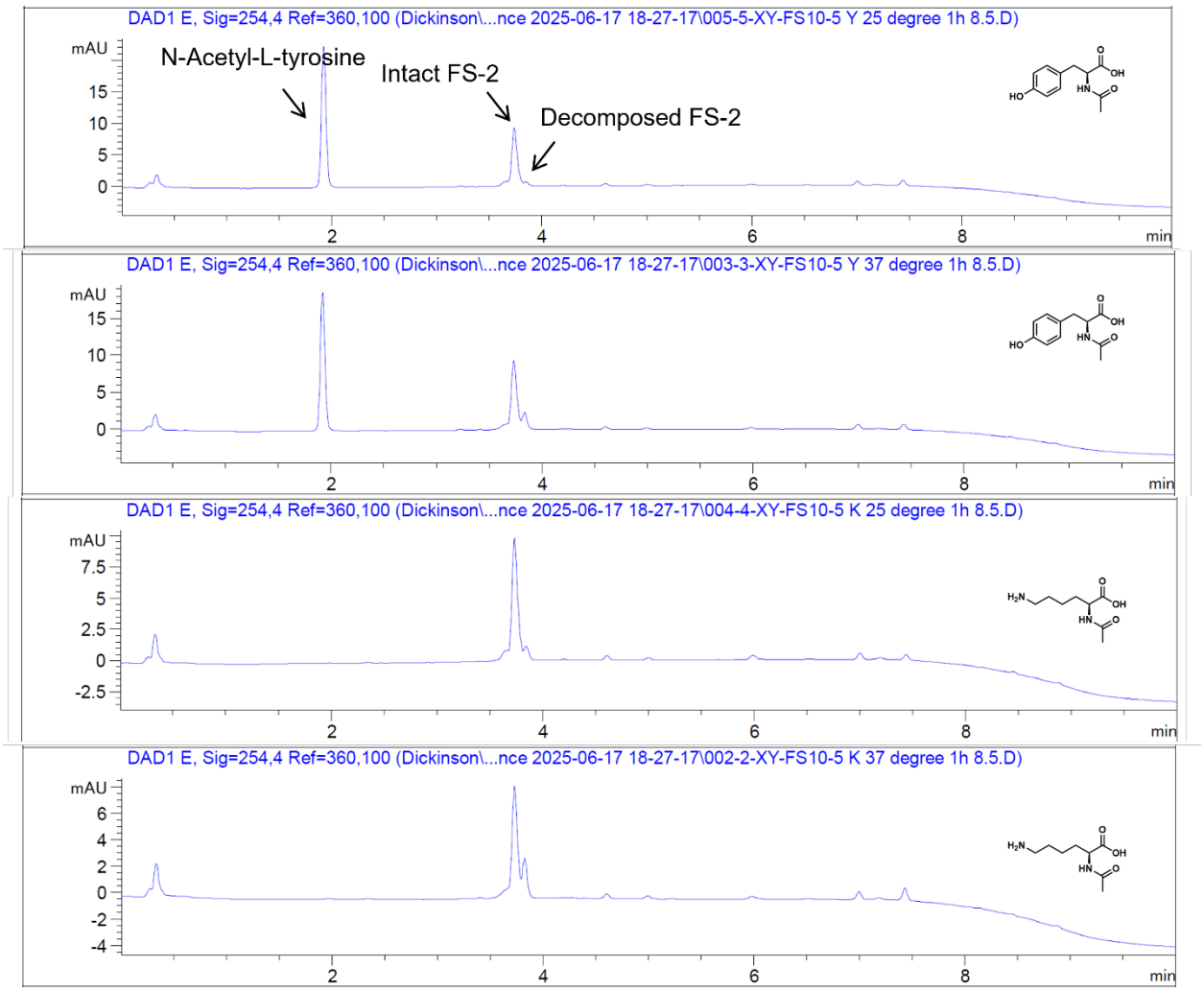


Figure S32. FS-2 exhibits minimal reactivity toward N-acetylated tyrosine or N-acetylated lysine (2 mM) following 1 h incubation at pH 8.5 and 25 °C or 37 °C.

SHAPE analysis for mapping peptide binding sites (Figure S33-S35)

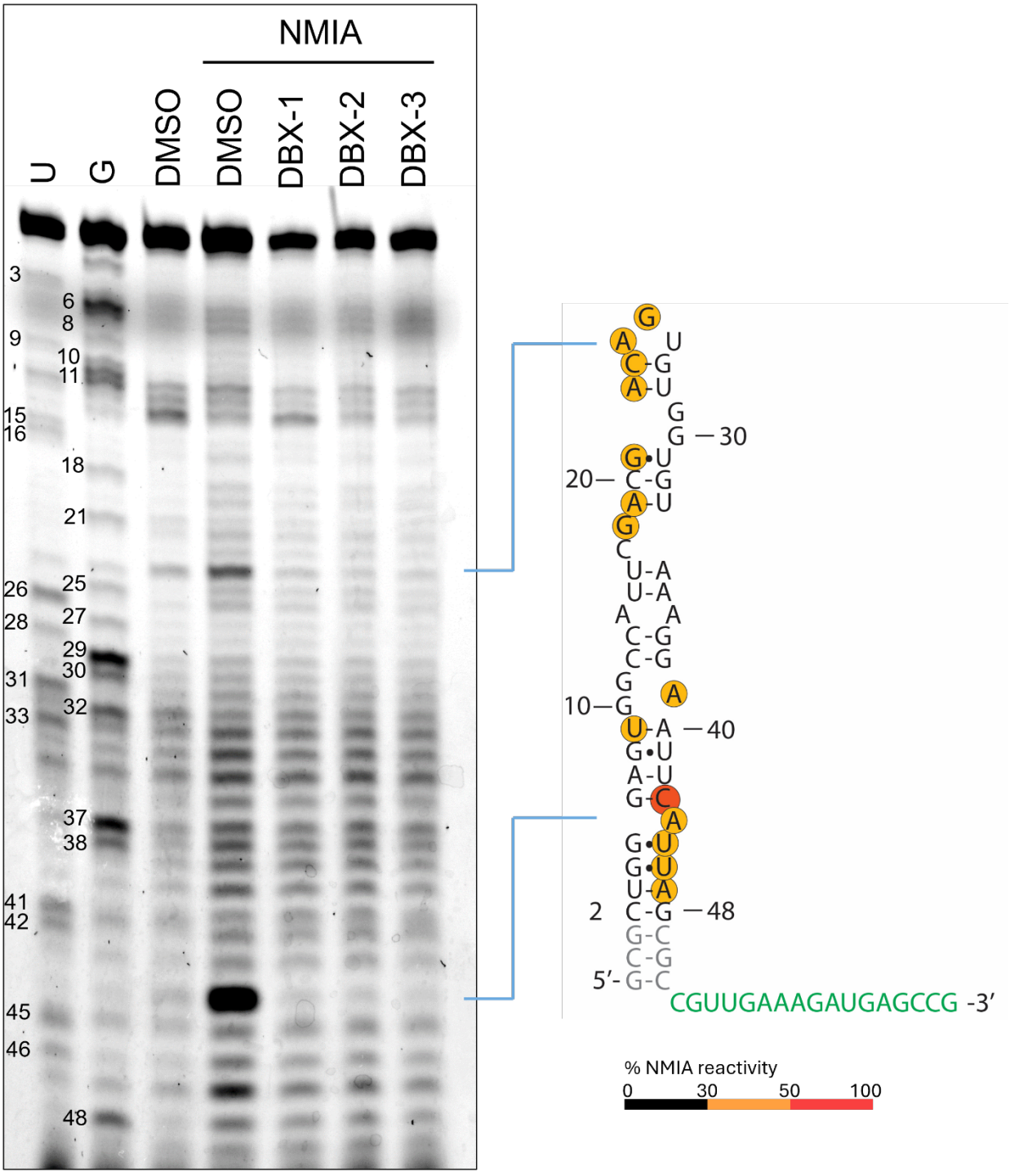


Figure S33. Peptide binding sites profiling with SHAPE using 2'-hydroxyl acylation probe, N-methylisatoic anhydride (NMIA). Representative SHAPE gel for three hit peptides (10 μM) from ***m*-DBX** cyclized library.

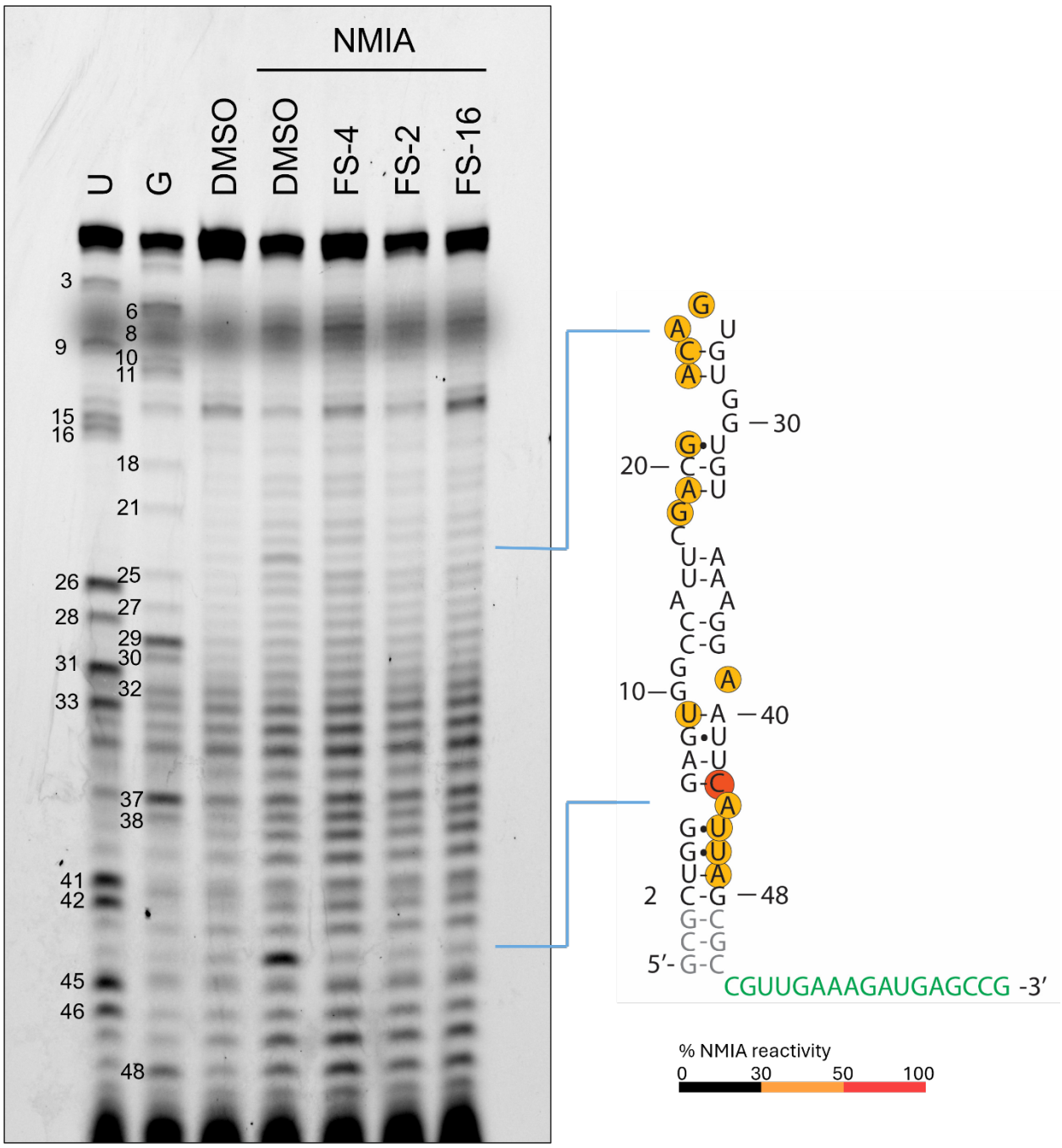


Figure S34. Representative SHAPE gel for three hit peptides from compound **2** (10 μM) cyclized library.

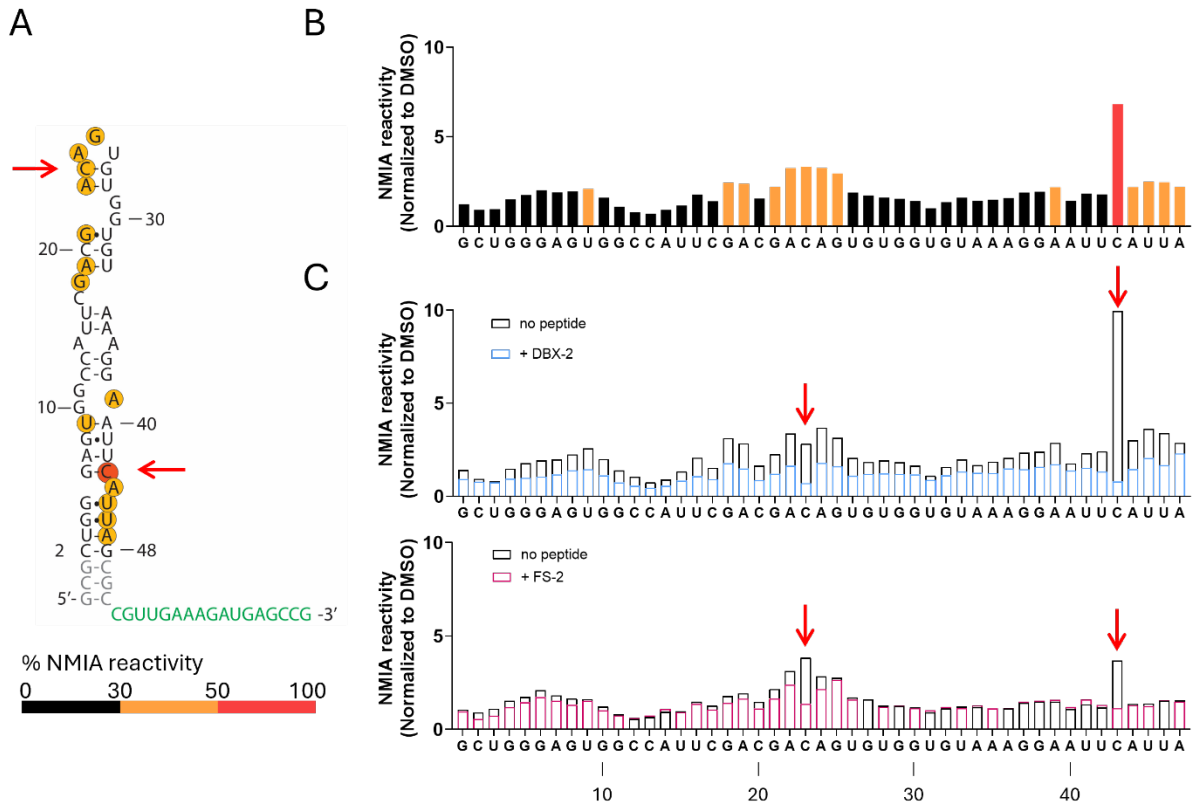


Figure S35. SHAPE analysis for *SNCA* IRE RNA with or without peptide incubated. Data are presented as modification efficiency as a function of nucleotide position. The most pronounced modification changes after incubation with DBX-2 or FS-2 are at nucleotides C43 and C23.

Predicted *SNCA* IRE model and two docking poses for FS-2 interaction with RNA (Figure S36)

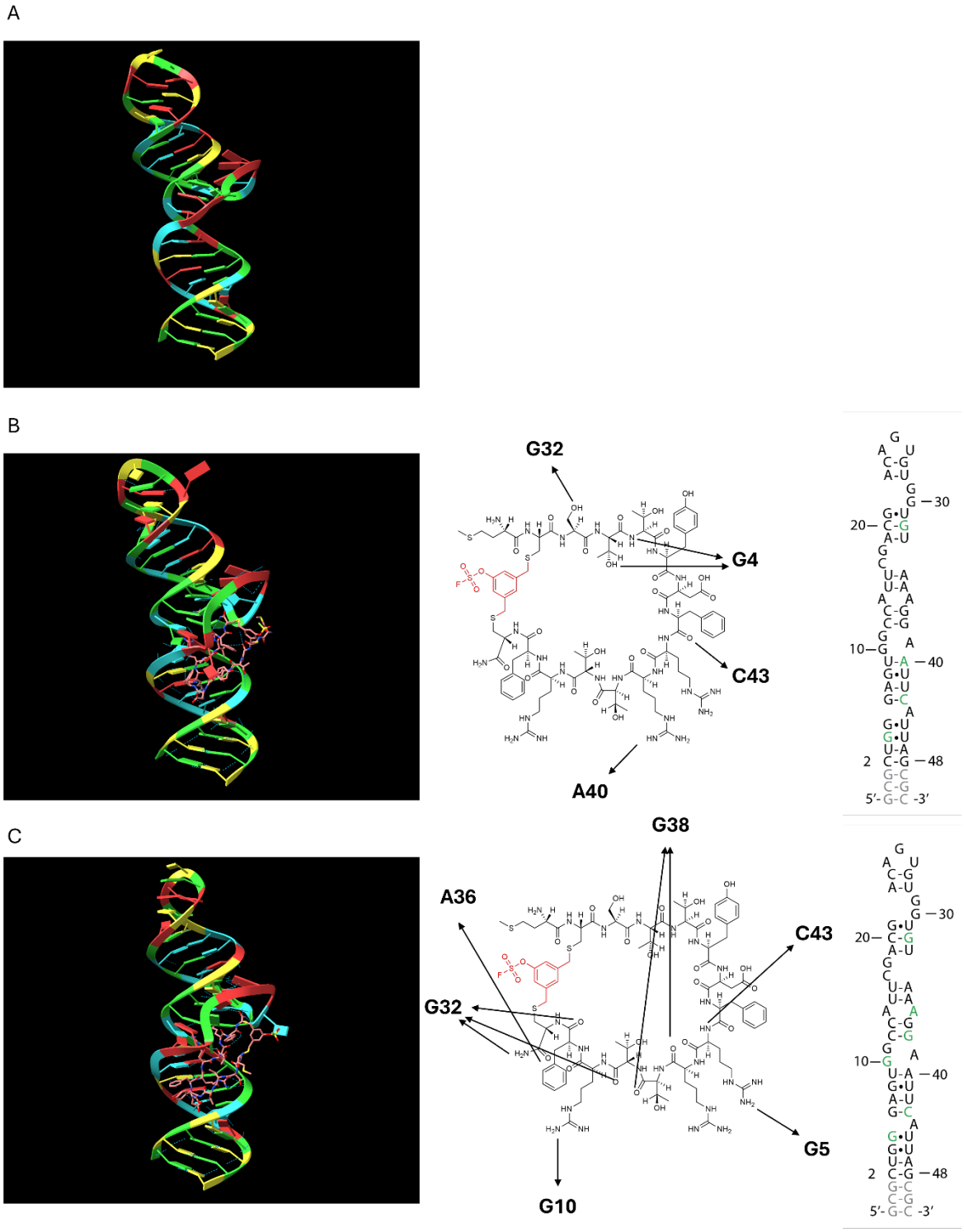


Figure S36. Docking poses predicted for FS-2 with *SNCA* IRE model. (A) Predicted unbound RNA model. (B, C) Two docking poses of FS-2 with the *SNCA* IRE model performed by Boltz-1. Hydrogen bonding interactions formed between cyclic peptide and nucleotides are highlighted on the right.

Denaturing PAGE gel analysis of reserve transcription stop signals triggered by covalent peptide (Figure S37)

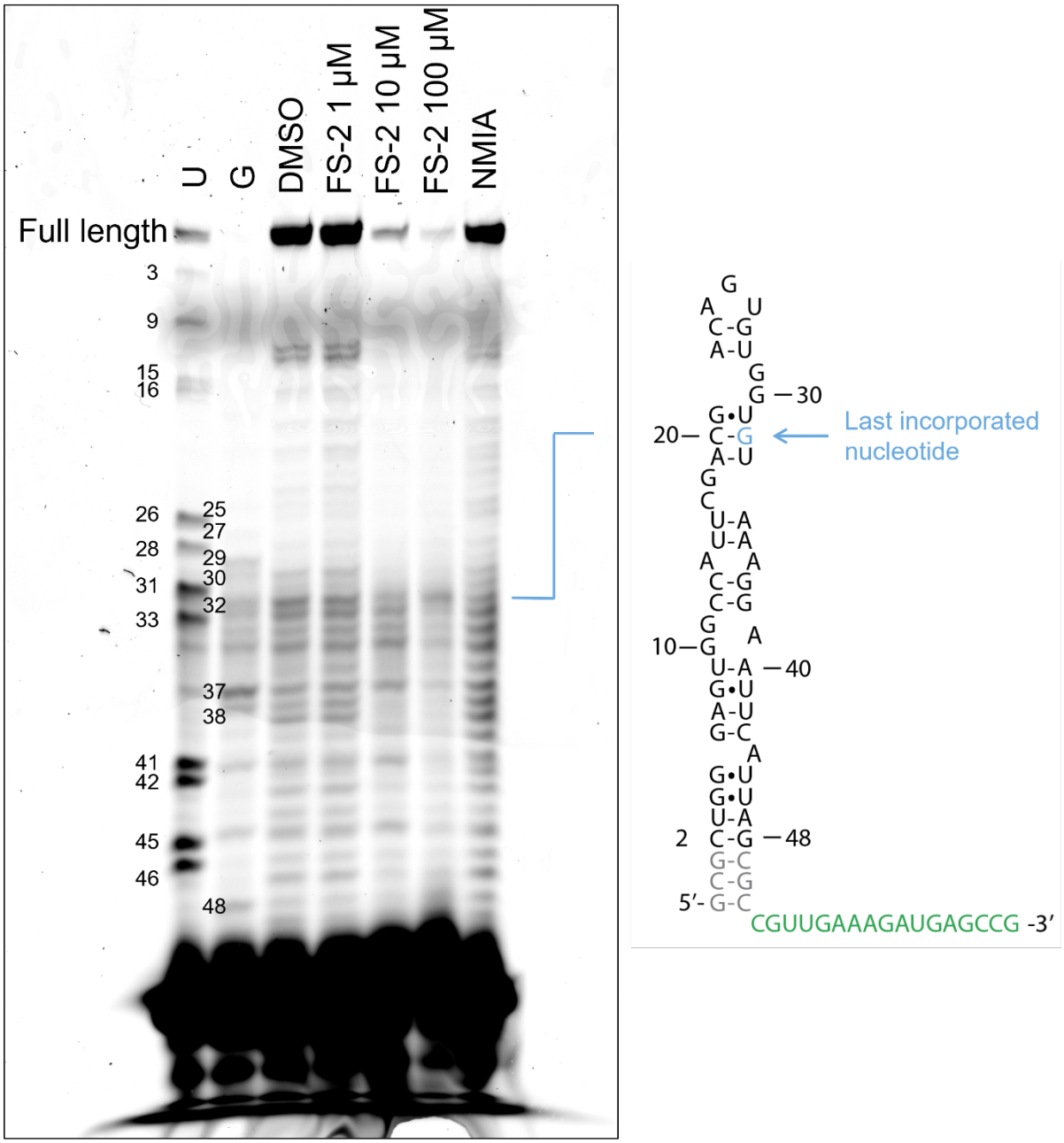
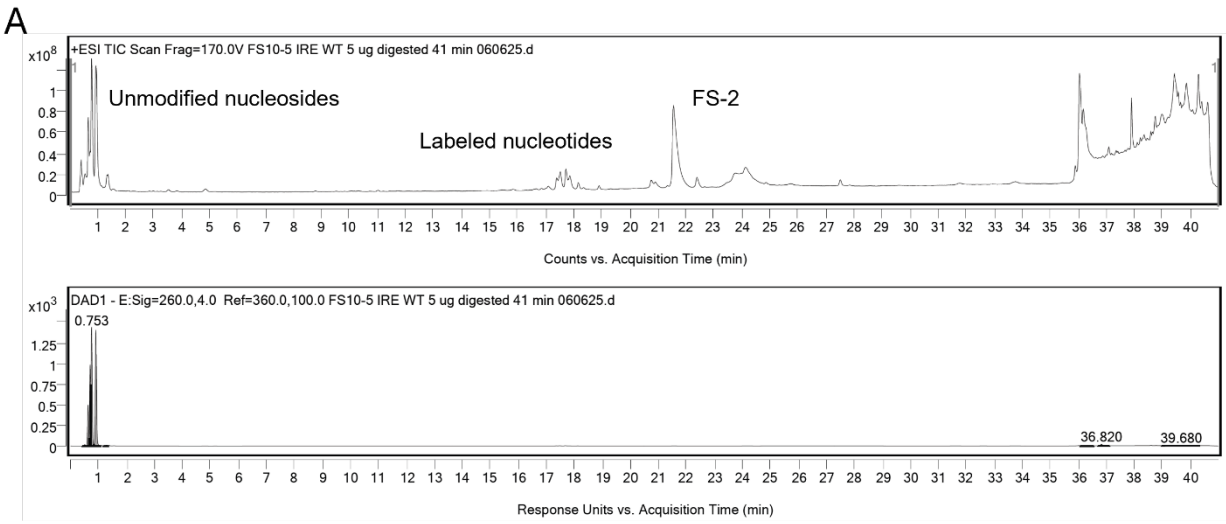
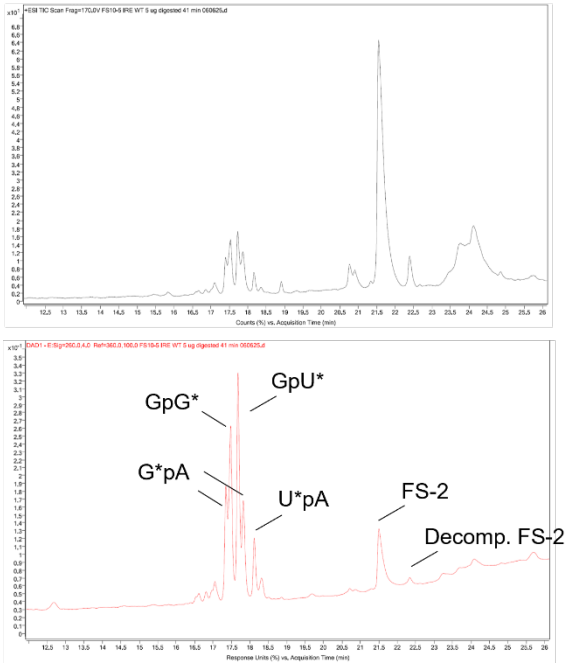


Figure S37. Denaturing PAGE analysis of reverse transcription stop sites for FS-2 treated *SNCA* IRE. RNA template was pre-incubated with FS-2 at 37 °C for 24 hours in 1× HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.5).

LC-MS/MS characterization of enzymatically digested FS-2 labeled *SNCA* IRE (Figure S38-44)

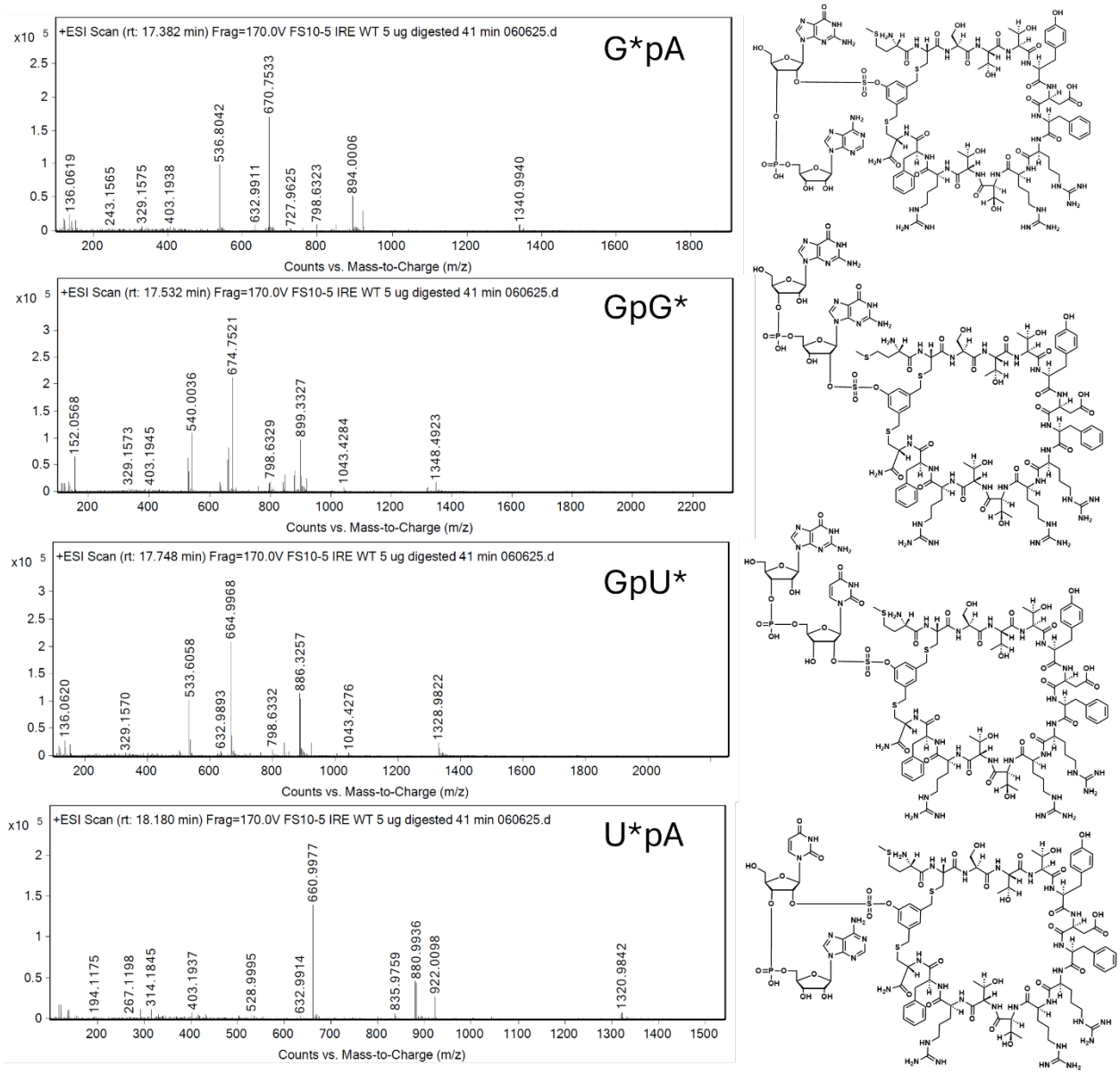




C

Ions	Retention time (min)
C	0.65-0.71
U	0.71-0.76
A	0.76-0.88
G	0.89-1.1
GpA	17.27-17.45, 17.82-18.04
GpG	17.51-17.64
GpU	17.67-17.80
UpA	18.06-18.28
FS-2	21.42-22.02
Decomp. FS-2	22.24-22.60

Figure S38. LC-MS analysis of digested RNA samples following FS-2 labeling. A) Total ion chromatogram (TIC) and UV trace monitored at 260 nm. B) Focused spectra (TIC and UV trace) showing FS-2 labeled dinucleotide products. C) Retention times of the identified labeled ions.



Ions	Calculated (m/z)				Observed (m/z)			
	2+	3+	4+	5+	2+	3+	4+	5+
G*pA	1340.4944	893.9987	670.7508	536.8021	1340.9940	894.0006	670.7533	536.8042
GpG*	1348.4919	899.3303	674.7496	540.0011	1348.4923	899.3327	674.7521	540.0036
GpU*	1328.9808	886.3230	664.9940	532.1967	1328.9822	886.3257	664.9968	533.6058
U*pA	1320.9834	880.9913	660.9953	528.9977	1320.9842	880.9936	660.9977	528.9995

* Denotes labeled nucleoside, sequence direction is not distinguishable

Figure S39. LC-QTOF analysis (positive ion mode) identified four FS-2 labeled dinucleotide adducts.

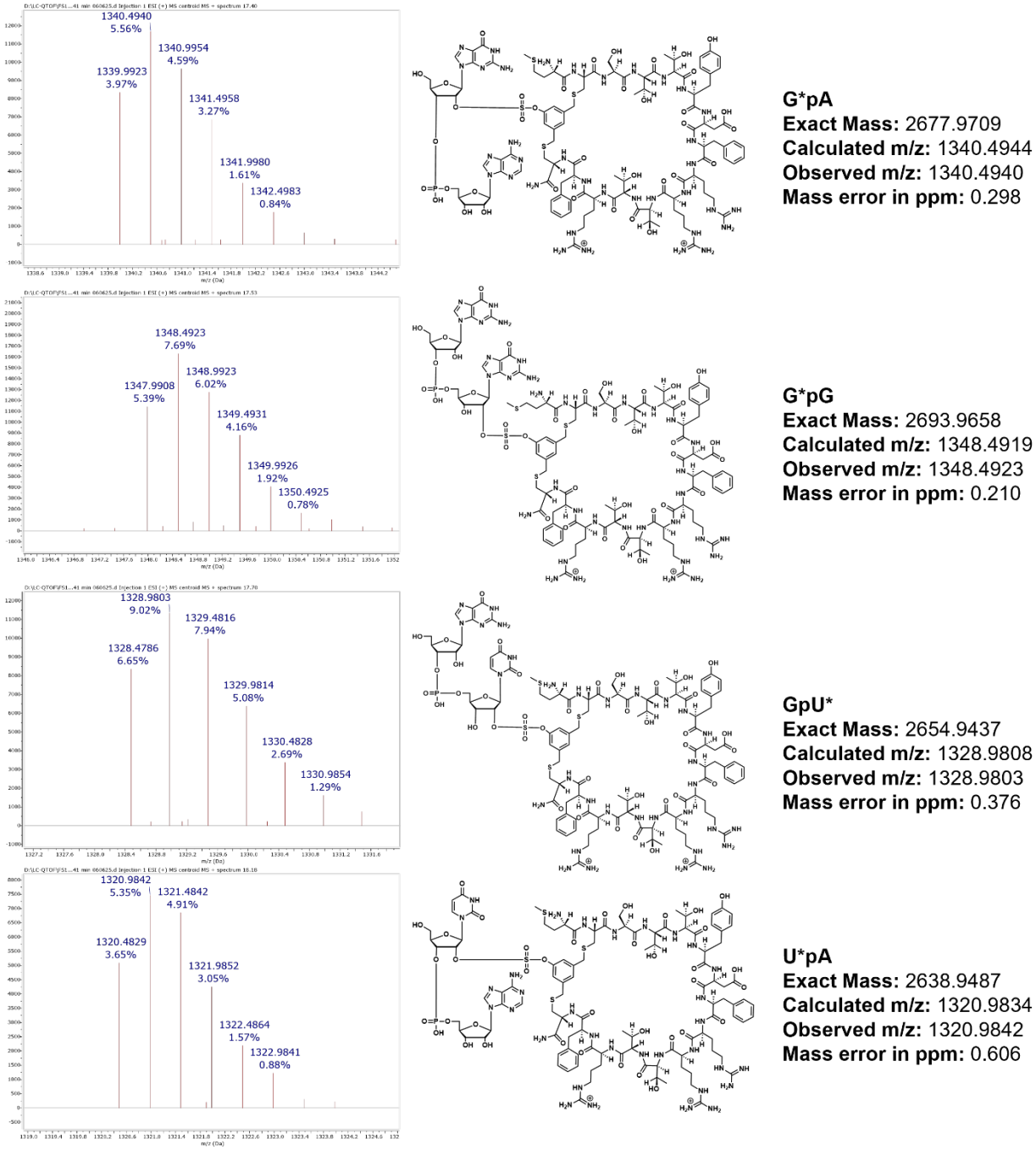
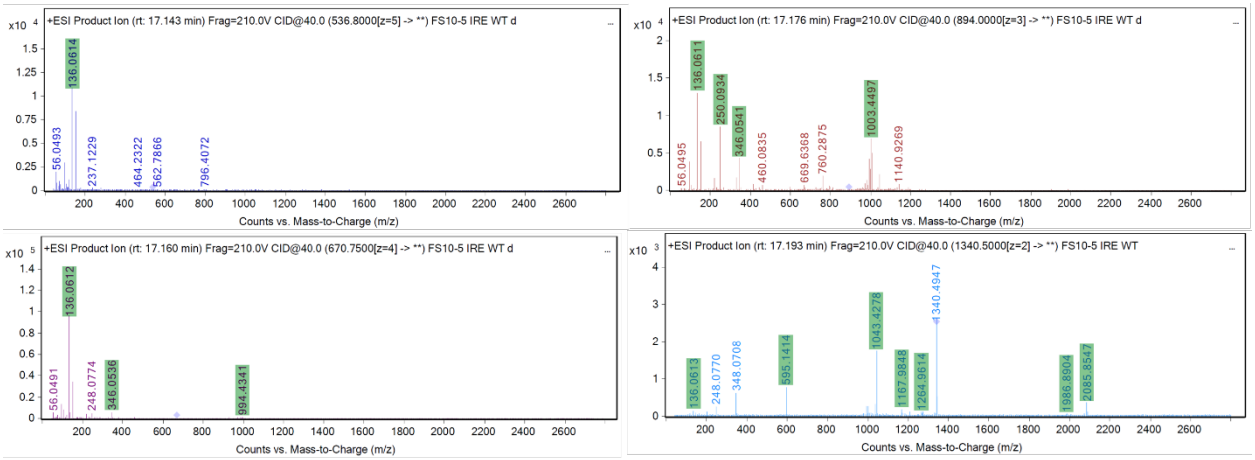


Figure S40. LC-QTOF analysis of the digested RNA sample revealed FS-2 labeled dinucleotide adducts exhibiting similar peptide isotopic distributions. The observed 2+ charged ions showed mass errors of less than 1 ppm from theoretical molecular weights.



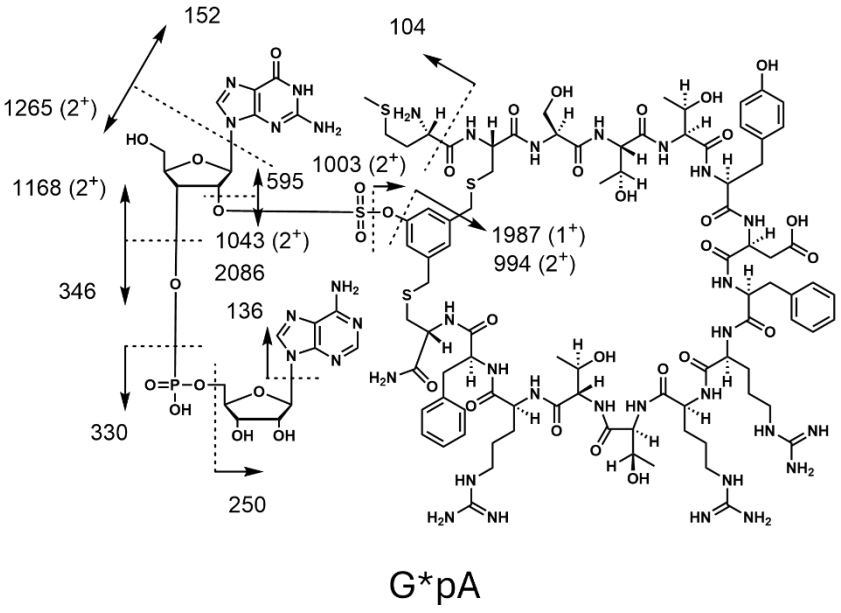
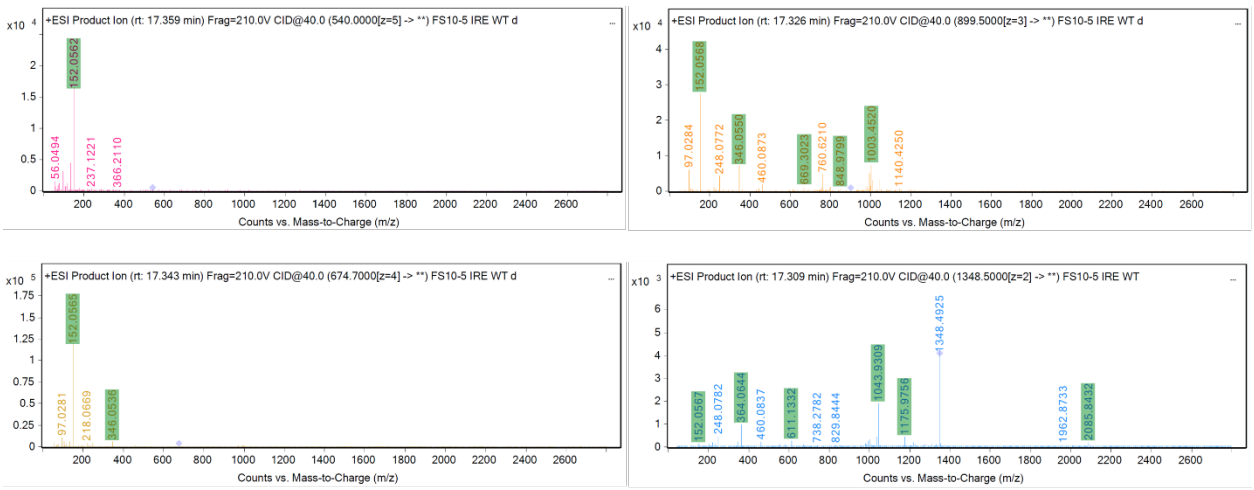


Figure S41. Targeted MS/MS analysis confirmed FS-2 labeling of G*pA (The asterisk denotes sulfonylation of the guanosine 2'-hydroxyl by the peptide). Strand orientation could not be distinguished.



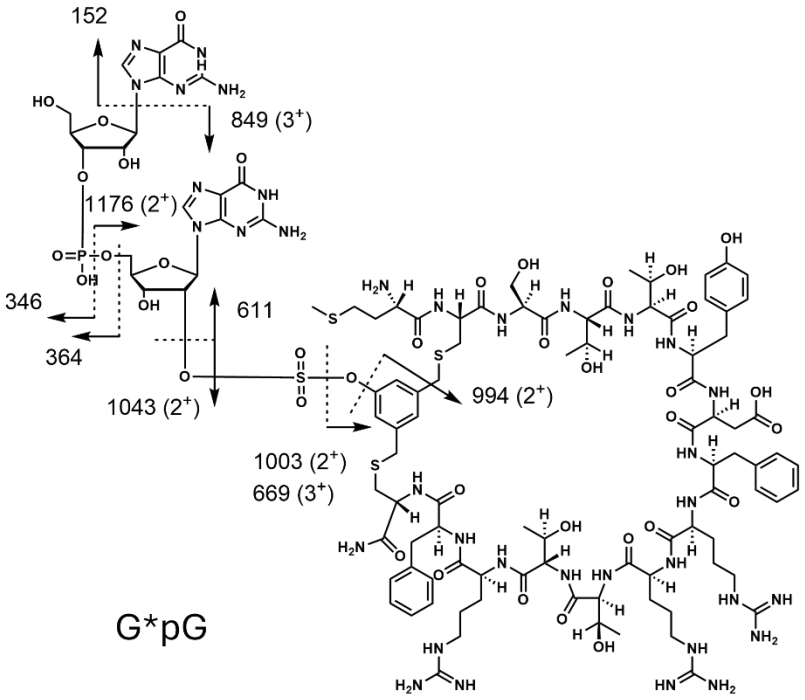
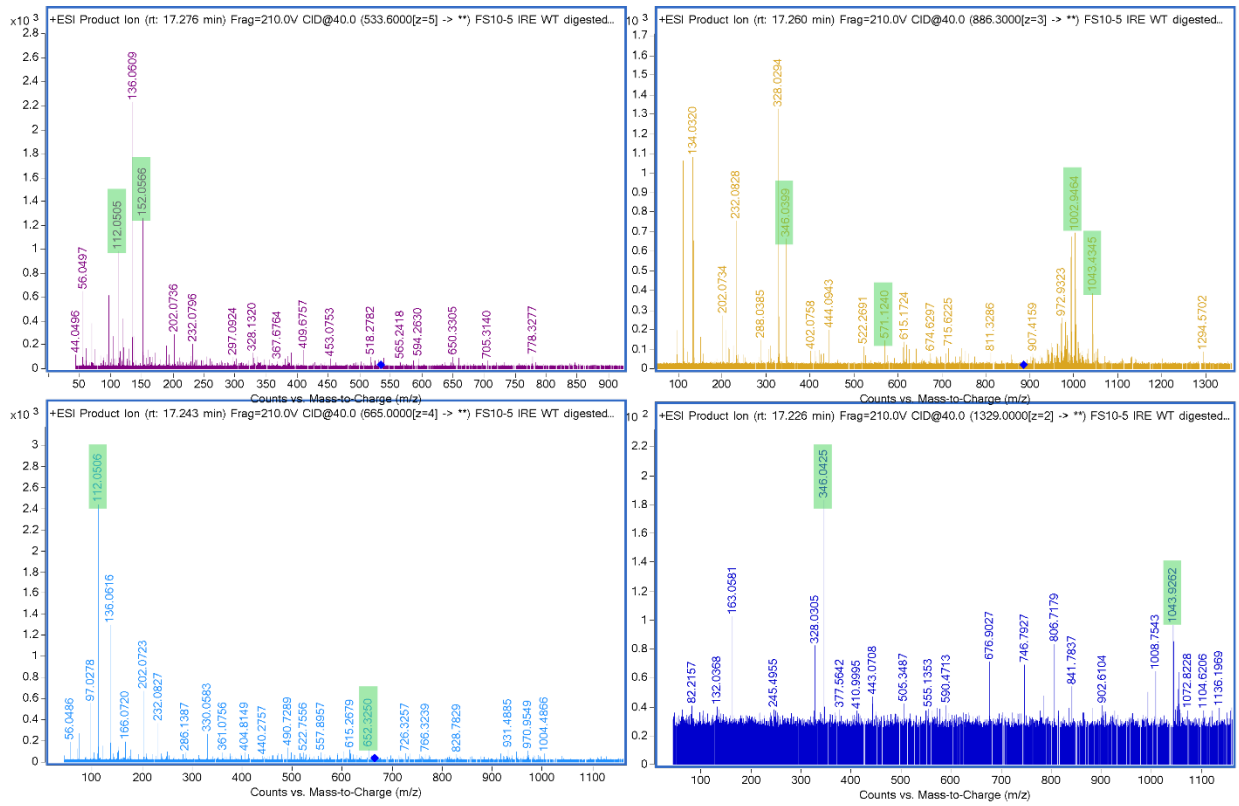


Figure S42. Targeted MS/MS analysis confirmed FS-2 labeling of G*pG (The asterisk denotes sulfonylation of the guanosine 2'-hydroxyl by the peptide). Strand orientation could not be distinguished.



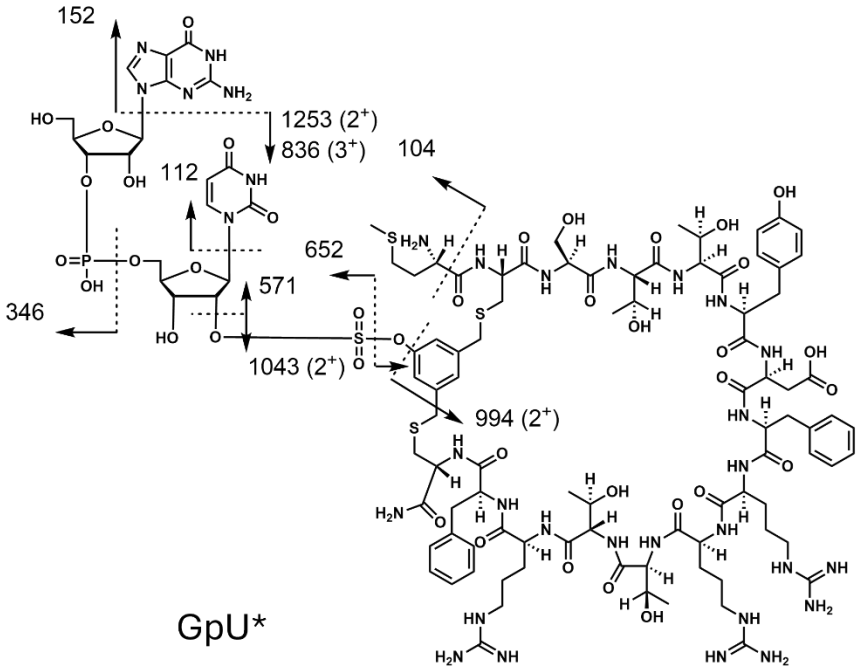


Figure S43. Targeted MS/MS analysis confirmed FS-2 labeling of GpU* (The asterisk denotes sulfonylation of the uridine 2'-hydroxyl by the peptide). Strand orientation could not be distinguished.

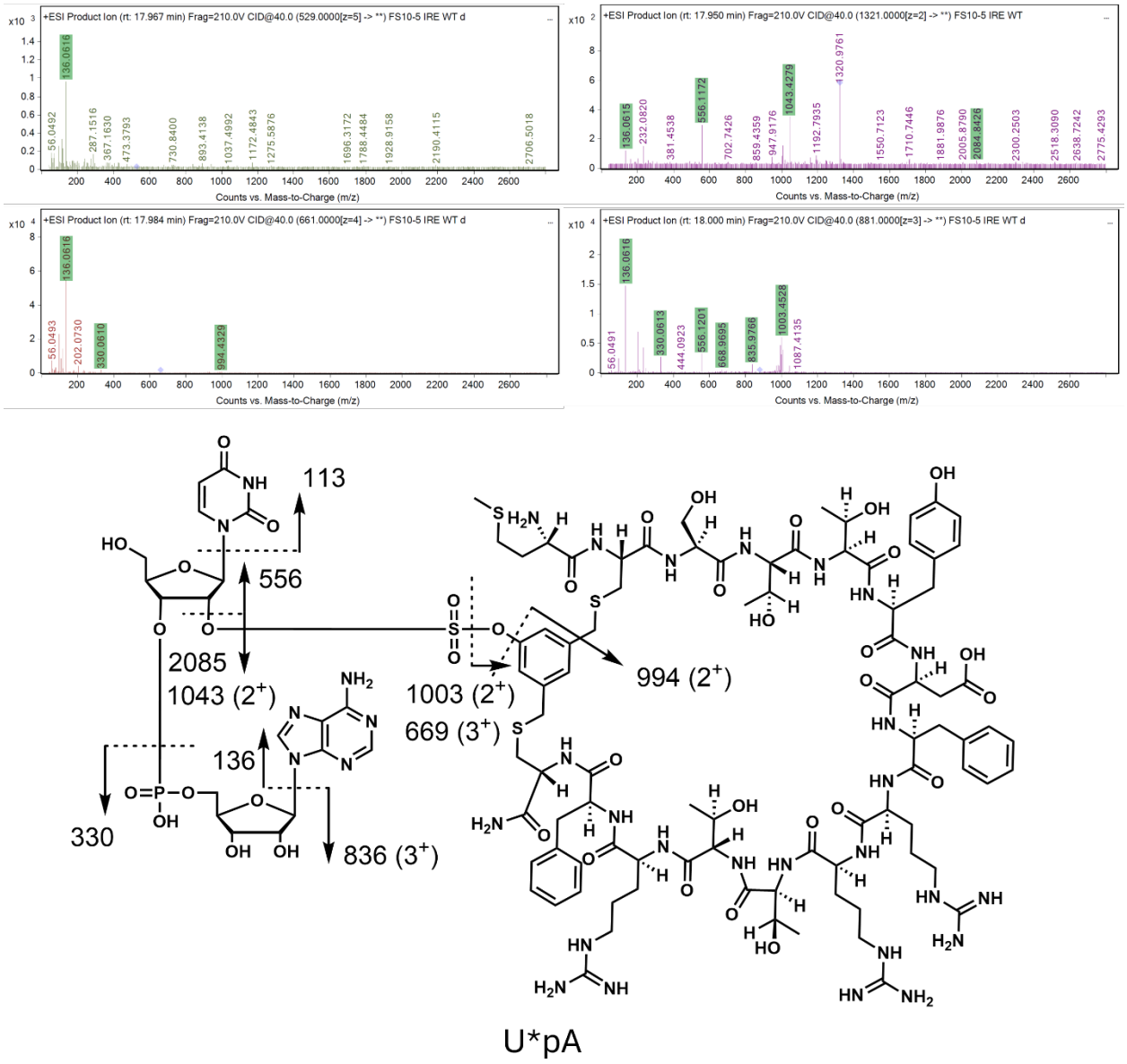


Figure S44. Targeted MS/MS analysis confirmed FS-2 labeling of U*pA (The asterisk denotes sulfonylation of the uridine 2'-hydroxyl by the peptide). Strand orientation could not be distinguished.

Experimental data for RNA cleavage by imidazole functionalized peptides (Figure S45-S47)

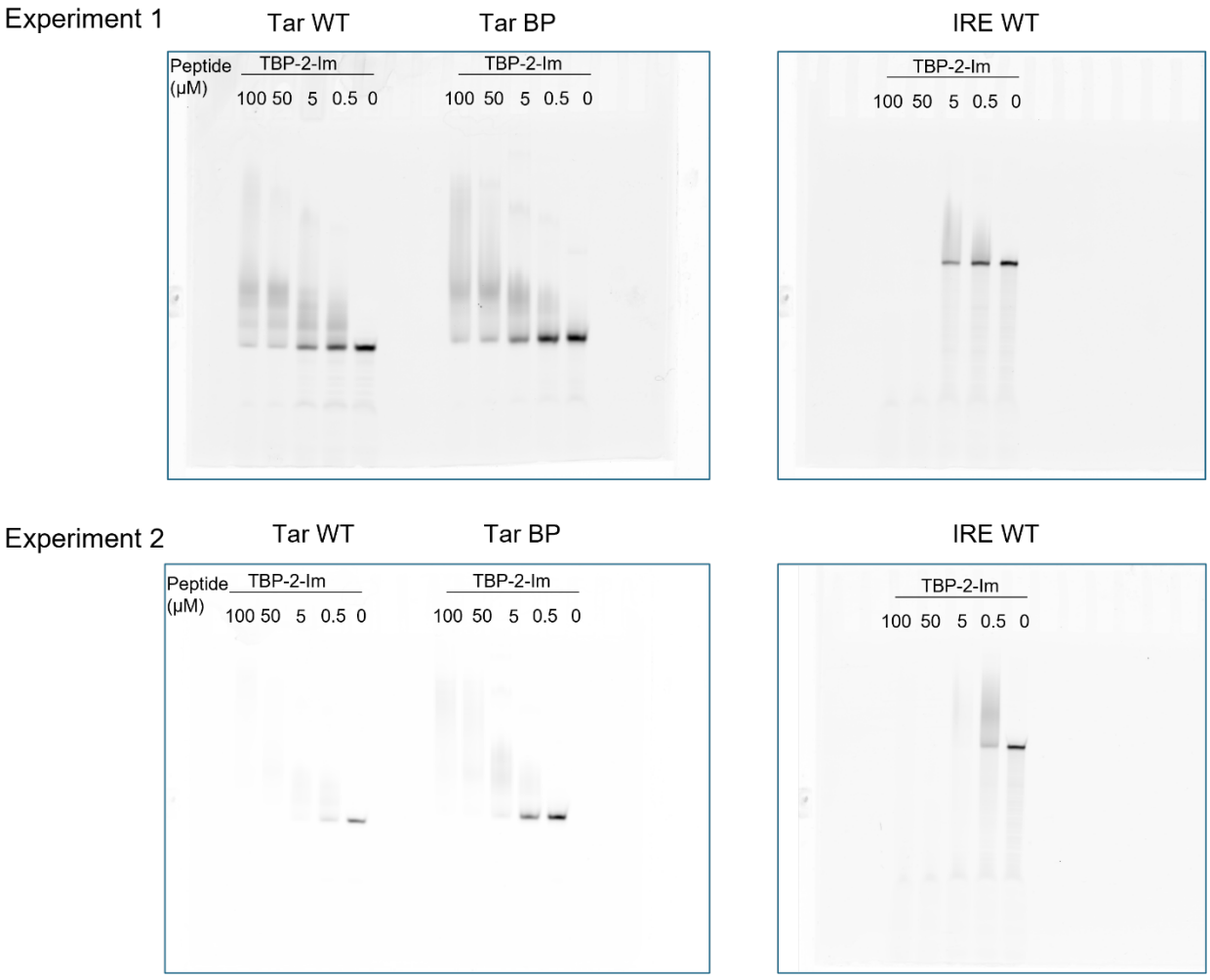


Figure S45. Denaturing 15% PAGE gel analysis of RNA degradation. RNA was incubated with TBP-2-Im at varying concentration at 37 °C for 6 hours in 1× HEPES buffer (25 mM HEPES, 10 mM $MgCl_2$, pH7.5). FITC-labeled RNA was detected at 492/508 nm (excitation/emission).

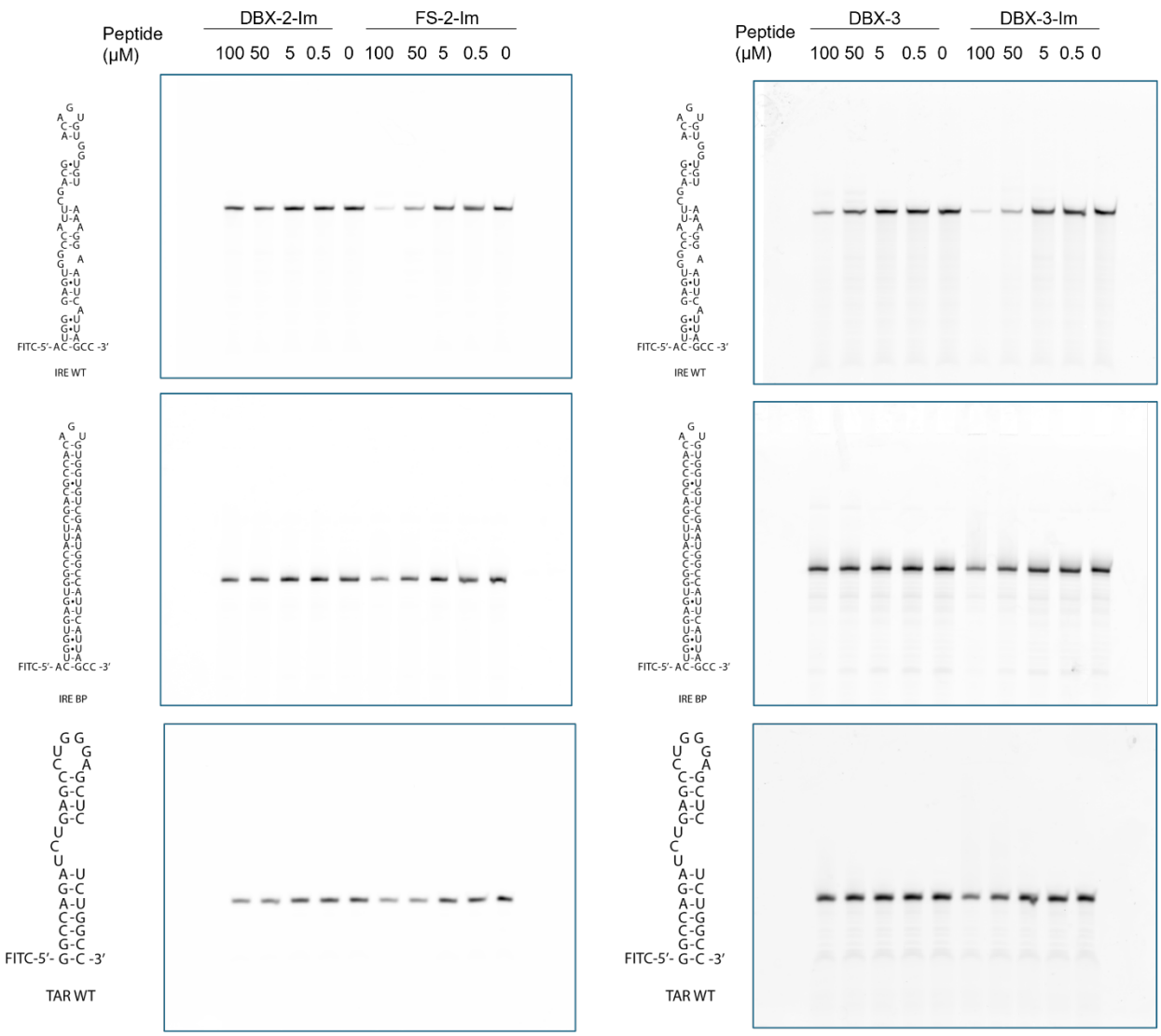


Figure S46. Denaturing 15% PAGE gel analysis of RNA degradation. RNA was incubated with peptides at varying concentration at 37 °C for 6 hours in 1× HEPES buffer (25 mM HEPES, 10 mM $MgCl_2$, pH7.5). FITC-labeled RNA was detected at 492/508 nm (excitation/emission).

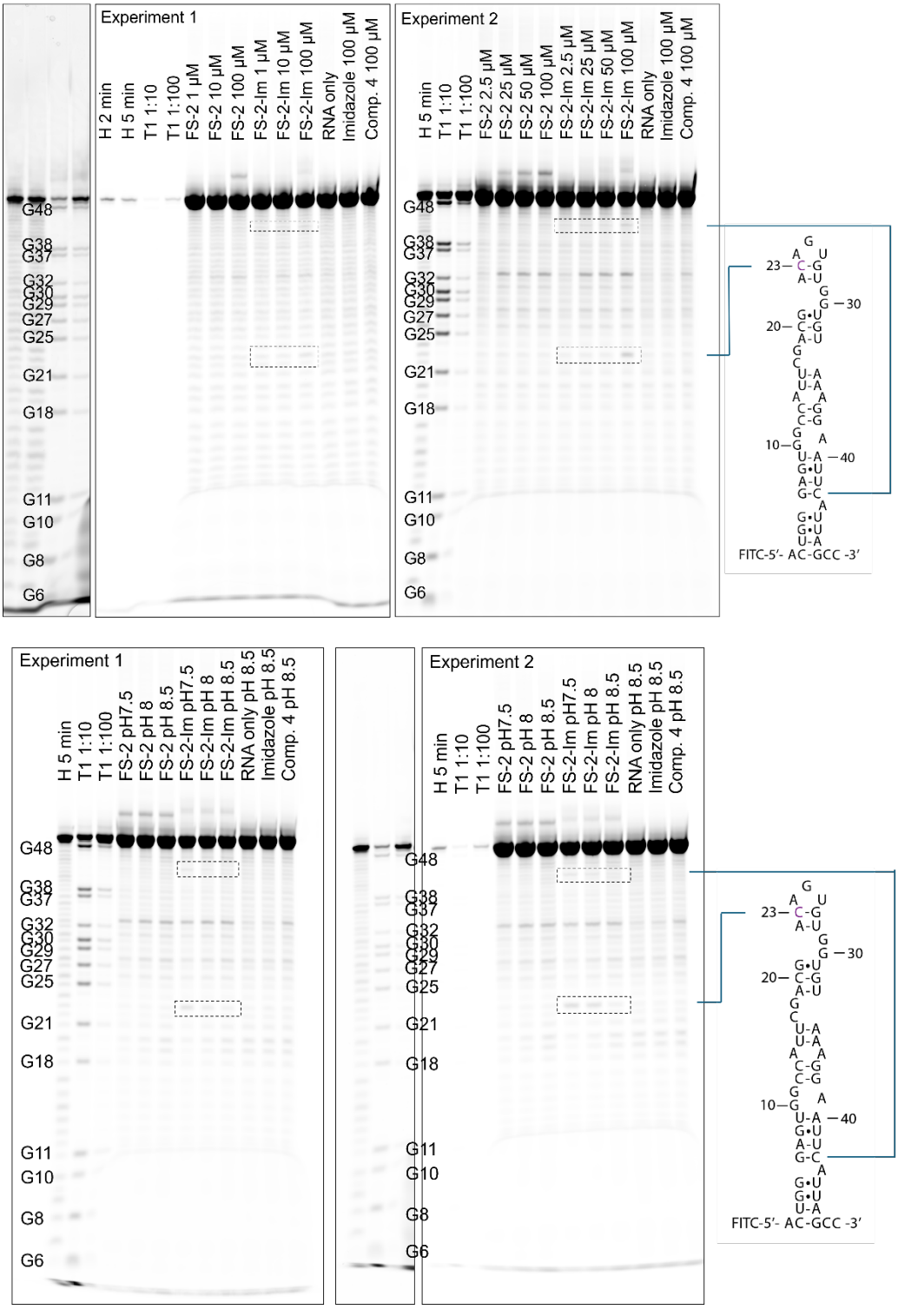


Figure S47. Denaturing 15% PAGE gel shows *SNCA* IRE degradation induced by FS-2-Im at varying concentrations and pH values.

FS-2-Im warhead stability and reactivity assessment (Figure S48-S50)

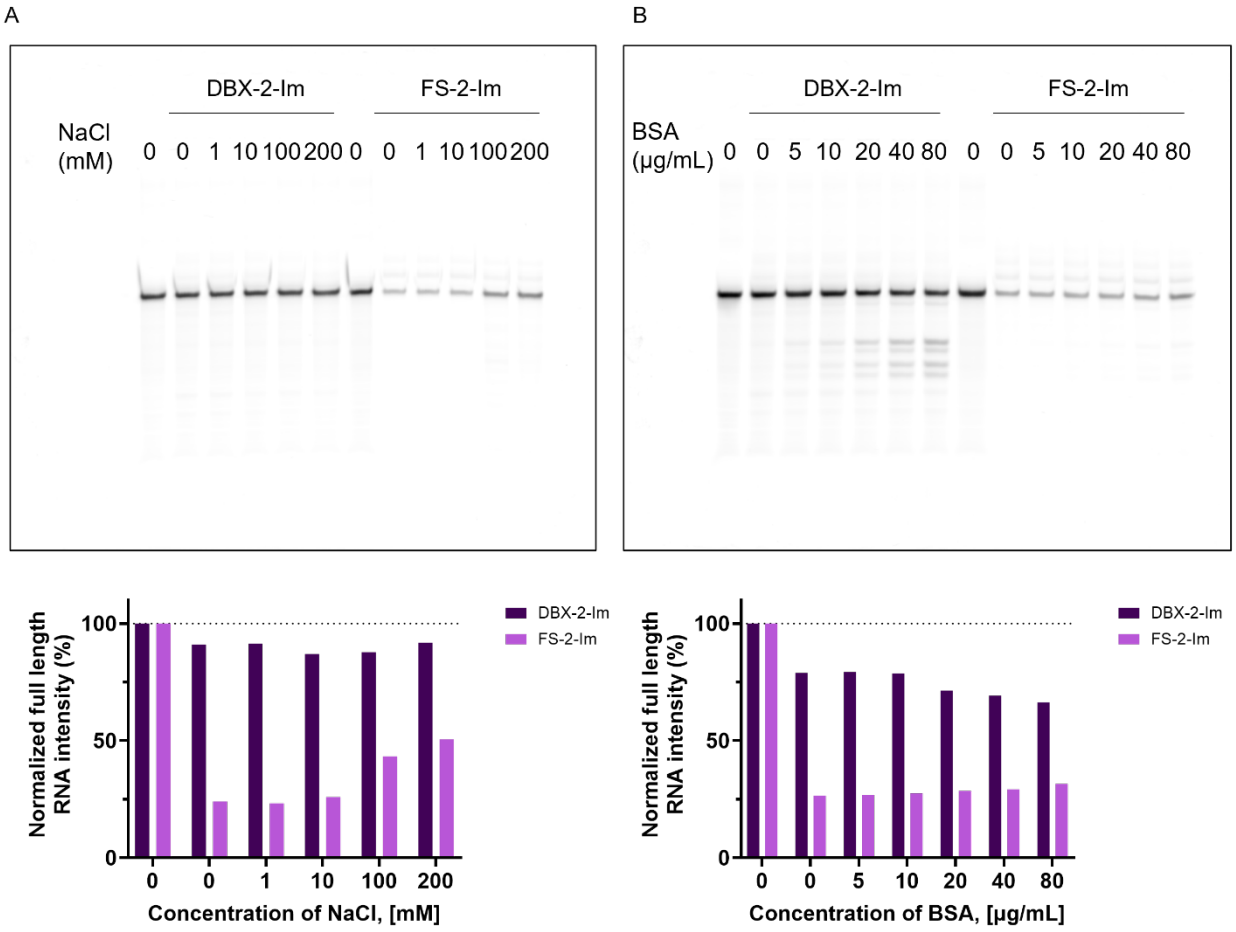
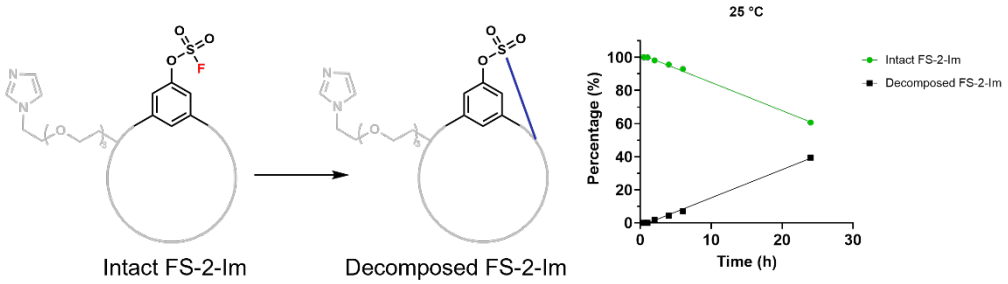
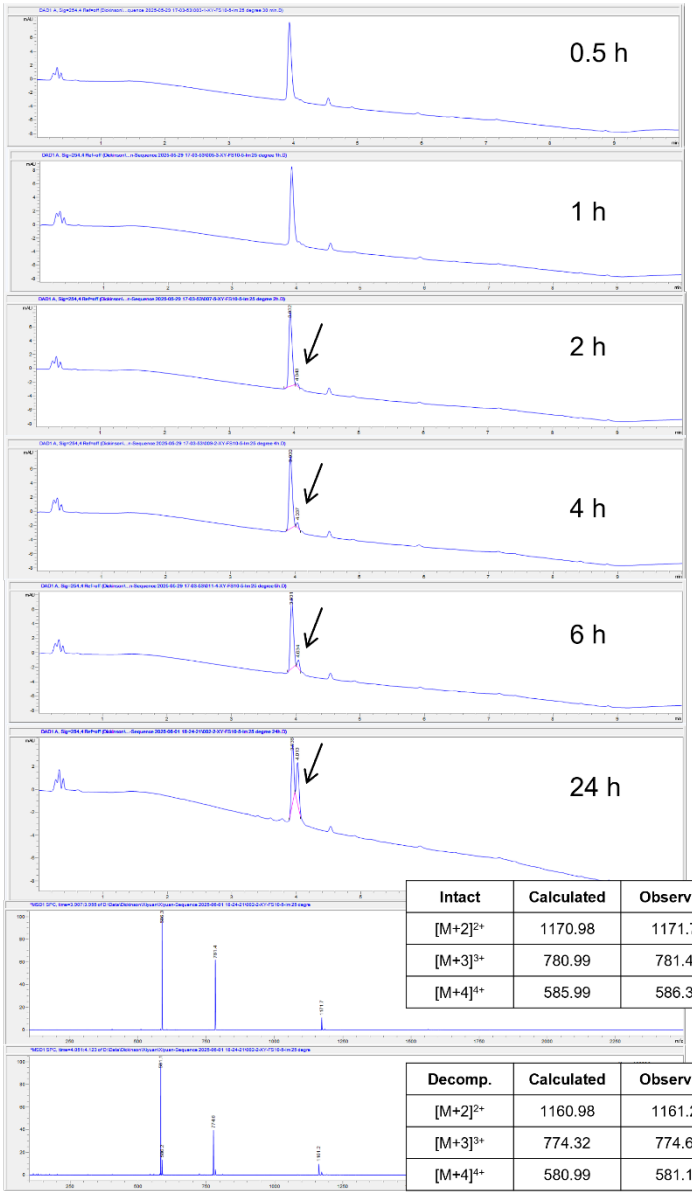


Figure S48. Effect of ionic strength and BSA additive on RNA degradation by DBX-2-Im and FS-2-Im. Reactions were performed at 37 °C for 6 hours in 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$. 500 nM RNA were incubated with 100 µM of DBX-2-Im or FS-2-Im. A) Increasing concentrations of NaCl resulted in reduced RNA degradation by FS-2-Im. B) Addition of BSA had minimal effect on degradation efficiency. (Higher concentration of BSA showed visible degradation likely due to RNase contamination).

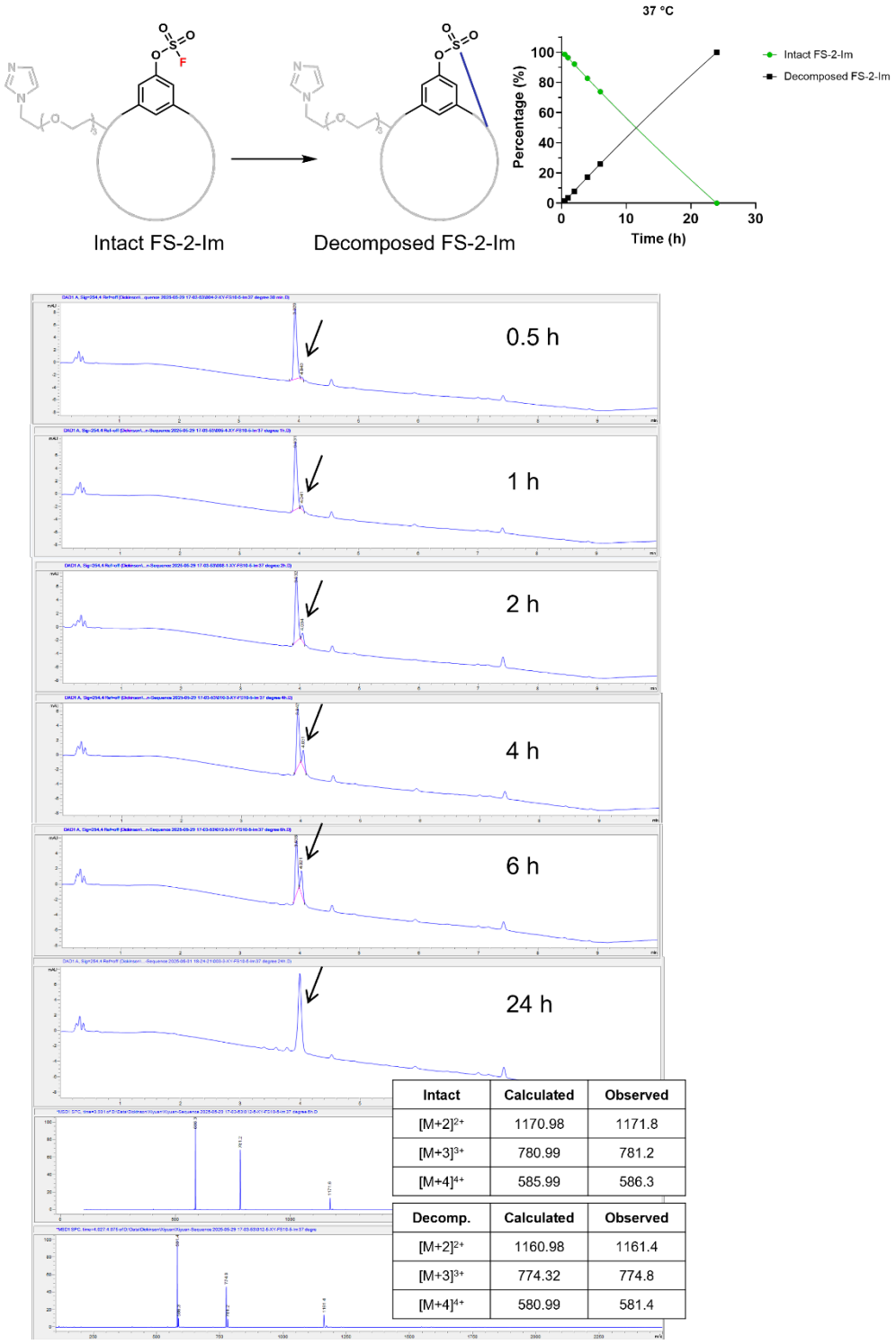




Intact	Calculated	Observed
$[M+2]^{2+}$	1170.98	1171.7
$[M+3]^{3+}$	780.99	781.4
$[M+4]^{4+}$	585.99	586.3

Decomp.	Calculated	Observed
$[M+2]^{2+}$	1160.98	1161.2
$[M+3]^{3+}$	774.32	774.6
$[M+4]^{4+}$	580.99	581.1

Figure S49. Stability test of FS-2-Im at 25 °C in HEPES buffer (pH 7.5).



Intact	Calculated	Observed
$[M+2]^{2+}$	1170.98	1171.8
$[M+3]^{3+}$	780.99	781.2
$[M+4]^{4+}$	585.99	586.3

Decomp.	Calculated	Observed
$[M+2]^{2+}$	1160.98	1161.4
$[M+3]^{3+}$	774.32	774.8
$[M+4]^{4+}$	580.99	581.4

Figure S50. Stability test of FS-2-Im at 37 °C in HEPES buffer (pH 7.5).

General procedures

^{1}H NMR, ^{13}C NMR, ^{19}F NMR spectra were recorded on Bruker Ascend 400 MHz instruments. Chemical shifts (δ) are reported as parts per million (ppm). ^{1}H NMR data are presented in the conventional format, including chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = quintet, m = multiplet, br = broad), coupling constants (Hz), and signal integrations. NMR spectra were analyzed using the MNova software package (14.2.0., Mestrelab Research, Santiago, Spain). In-line LC-ESI-MS analysis was performed on an Advion LC System (AVANT 2012) coupled with an Advion Expression-L mass spectrometer (Ithaca, NY). Mass spectra were recorded in positive-ion mode and negative-ion mode with a capillary voltage of 4.5 kV over a mass-to-charge scan range of 100–2000 m/z. Compound purity was determined in methanol or acetonitrile containing 0.1% formic acid and separation of the diluted samples on a 2.1 mm × 100 mm reversed-phase Kinetex 1.7 µm C18 100Å analytical column (00D-4475-AN) at 40°C using the following solvent system: solvent A, Optima water/ 0.1% formic acid, and solvent B, methanol/0.1% formic acid, at a flow rate of 0.3 mL/min and a gradient of 5% B for 3 min, 5% B→100% B over 6 min, 100% B for 4.9 min, 100% B→5% B over 0.1 min followed by 5% B over 2 min. Elution profiles were recorded at four wavelength at 214 nm, 254 nm, 280 nm, 364 nm. High-resolution mass spectra for compounds were obtained on an Agilent 6224 TOF using a combination of atmospheric pressure chemical ionization and electrospray (APCI) and electrospray ionization (ESI). Matrix-assisted laser desorption/ionization (MALDI) analysis was carried out on the Bruker Autoflex Max MALDI-TOF-TOF using α-Cyano-4-hydroxycinnamic acid as matrix.

Model peptide cyclization and detection by MALDI-TOF

100 µM of somatostatin or TAR binding peptides (custom synthesized from GenScript, Piscataway, NJ) (1 eq.) was dissolved in 1× Tris buffer (20 mM Tris HCl, pH 7.8) containing 200 µM tris(2-carboxyethyl)phosphine (TCEP) and 30% acetonitrile. A fully dissolved linker (20 eq.) in acetonitrile was then added to achieve a final concentration of 2 mM. The reaction mixture was incubated at room temperature for 1 hour and monitored by ESI-LS-MS. Upon completed, the sample was analyzed by MALDI-TOF MS using positive ion mode. Adrenocorticotropic hormone (ACTH) fragment 18-39 was used as an external standard.

mRNA display

Procedures for PCR, *in vitro* transcription and *in vitro* translation were adapted from published protocols[1]. All reagents used in this study are listed in **Table S1**. DNA templates encoding randomized peptide libraries of 6, 8, 10, or 12 amino acids were individually amplified by PCR and subsequently transcribed into RNA, which was purified using the RCC-25 kit. The purified RNA templates were then ligated to a puromycin-conjugated DNA oligonucleotide (**Table S2**) and purified by 10% denaturing PAGE. An equal amount (150 ng) of ligated products from each library was pooled and subjected to *in vitro* translated at 37 °C for 30 minutes using PURExpress Δ RF123 Kit. Following the translation reaction, $MgCl_2$ and KCl were added to achieve final concentration of 25 mM and 300 mM, respectively, and the reaction was further incubated for an additional 15 minutes at 37 °C. The efficiency of in *vitro* translation was evaluated by denaturing SDS-PAGE (8% resolving gel with a 4% stacking gel. Fluorescence signals were detected at 498/517 nm (excitation/emission).

Purification with HA tag

Translated products expressing the HA peptide were captured by anti-HA magnetic beads. After *in vitro* translation, the solution was diluted with 1X TBST buffer (25 mM Tris HCl, pH 7.5, 0.05% Tween-20) and 8 volumes of pre-equilibrated anti-HA magnetic beads (washed twice with TBST buffer) were added. The mixture was incubated at room temperature for 30 minutes. After incubation, the supernatant was discarded, and the beads were washed twice with 1X TBST buffer and once with 1X TBS buffer (25 mM Tris HCl, pH 7.5). For elution step, the beads were suspended in 11 µL of 5X RT buffer (PrimeScript buffer, Takara), 33.6 µL of water and then supplemented with 11 µL of HA peptide (YPYDVPDYA) to a final concentration of 2 mg/mL. After incubation at room temperature for 1 hour, the eluted product was collected and subjected to reverse transcription.

Reverse transcription

Complementary DNA (cDNA) was generated from the purified translation product via reverse transcription. The eluted translation product was combined with 2.9 µL of PrimeScript RT enzyme mix and 2.9 µL of a 100 µM RT primer. The reverse transcription was performed at 42 °C for 30 minutes. To establish a standard curve for the quantitation of recovered cDNA from each round of selection, an equal amount of ligated RNA (600 ng) was subjected to reverse transcription without performing translation or HA beads purification. Following the RT step, a series of dilute solution (1:10) was prepared using nuclease-free water, and the corresponding Cq values obtained from quantitative PCR (qPCR) were used to generate the standard curve.

Oligo-$(dT)_{25}$ beads cleanup and cyclization

Oligo-$(dT)_{25}$ magnetic beads were pre-washed twice with 500 µL of Lysis/Binding buffer (100 mM Tris pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) and resuspended in 100 µL of Lysis/Binding buffer. The resuspended beads were then added to the reaction mixture and rotated at room temperature for 15 minutes.

After the incubation, the supernatant was discarded, and the beads were washed twice with Washing Buffer B (10 mM Tris, pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.2 mM TCEP). Then the beads were subsequently resuspended in Tris buffer (25 mM Tris, pH 7.8, 20 µM TCEP) and subjected to two times elution by heating at 50 °C for 2 minutes.

The combined solution was adjusted to 33% acetonitrile and supplemented with 1 mM *m*-DBX or compound **2**. The cyclization reaction was carried out at room temperature for 1.5 hours. Following incubation, a second round of Oligo-$(dT)_{25}$ beads purification was performed to remove excess linker and exchange the buffer to RNA folding buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$). After this step, 15 µL of 100 µL elution solution was retained for quantification of cDNA recovery by qPCR.

Selection with immobilized RNA

A total of 500 pmol of 5' end biotinylated *SNCA* IRE was folded in RNA folding buffer by heating at 90 °C for 3 minutes, followed by cooling on ice for 15 minutes. The folded RNA was then immobilized on 500 µg of pre-washed streptavidin magnetic beads (10 mg/mL) (Pierce) at room temperature for 30 minutes. After immobilization, the RNA bound beads were washed twice with washing buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mg/mL BSA) and resuspended in 50 µL of RNA folding buffer. To minimize non-binders and nonspecific bindings to beads, an equal amount of streptavidin beads without RNA immobilization was prepared in the same manner and used for negative selection.

Both RNA immobilized beads and empty beads were resuspended in blocking solution (25 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mg/mL BSA, 0.1 mg/mL tRNA) and incubated at 4 °C for 30 minutes prior to library incubation.

Following the blocking step, empty beads were first used for negative selection by incubating with the library in blocking solution, after which the supernatant was collected and subjected to a second step of negative selection with empty beads or directly incubated with RNA-immobilized beads. Positive selection was performed at room temperature for 1 hour in the presence of fully base-paired target RNA as a competitor. The concentration of competitors and modifications made across 10 rounds of selection are listed in **Table S3**.

After the incubation, the streptavidin beads were washed 2-3 times with washing buffer and once with RNA folding buffer. The bound cDNAs were eluted using 100 µL of TBS buffer by heating at 95 °C for 5 minutes.

Table S3. Experimental conditions used to increase selection stringency.

Round	1	2	3	4	5	6	7	8	9	10
Negative selection	No	1×	1×	1×	2×	2×	2×	2×	2×	2×
Target (pmol)	500	500	500	500	500	500	400	400	300	300
Positive selection temp. (°C)	4	4	25	25	25	25	25	25	25	25
Positive selection time (h)	20	20	1	1	1	1	1	1	1	1
Wash (times)	3	3	3	3	4	4	4	4	4	4
Base-paired RNA (pmol)	–	–	–	–	2500	2500	2500	2500	2500	2500

Determination of cDNA recovery by qPCR

To quantify the cDNA recovered from each round of selection and monitor selection progress, qPCR reactions were performed (three technical replicates) using PowerUp™ SYBR™ Green Master Mix (Thermo) at 20 µL reaction scale. The experiments were carried out on a MicroAmp™ Optical 384-Well Reaction Plate (Thermo) with Applied Biosystems QuantStudio 6 Pro System. The primers used are listed in **Table S2**. ROX was set as a passive reference dye to normalize SYBR green fluorescence signals. The cDNA concentration (C) (ng/µL) was determined using the standard curve, as described above. The percentage of cDNA recovery was calculated using **equation 3**:

$$cDNA\ recovery\ (\%) = 100 \times \frac{C_{after\ selection} \times 100\ \mu L}{C_{before\ selection} \times 85\ \mu L}$$

PCR amplification to generate DNA library for next round

The eluted cDNA was amplified using Q5 High Fidelity DNA Polymerase according to the manufacturer's protocol, with the following modifications: Initial denaturing step: 98 °C for 60 sec; followed by cycles of denaturing step: 98 °C for 20 sec; Annealing step: 68 °C for 30 sec; Elongation step: 72 °C for 40 sec. A final elongation step was 72 °C for 5 minutes. The number of amplification

cycles was determined by the Cq value acquired from the qPCR step, plus an additional 2-5 cycles. Following amplification, PCR products were purified using QIAquick PCR purification Kit (Qiagen) and analyzed with 2% agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 200V for 40 minutes.

To include DNA adaptors for high throughput sequencing analysis, primers with adaptor regions were used for PCR (**Table S2**). The concentrations of cDNA template were measured using dsDNA HS Qubit assay and the final concentrations were adjusted to 3-4 ng/μL. The PCR was performed using the following method: Initial denaturing step: 98 °C for 5 min; followed by cycles of denaturing step: 98 °C for 15 sec; Annealing step: 68 °C for 15 sec; Elongation step: 72 °C for 30 sec; A final elongation step was 72 °C for 5 min. The number of amplification cycles was 13. After purifying the amplified DNA with QIAquick PCR purification Kit, the sample concentrations were measured and normalized with Qubit assay. Samples were sequenced using Amplicon EZ sequencing service (Genewiz, South Plainfield, NJ).

Data analysis

In silico translation and sequence clustering

Sequencing results (.fasta file) were processed with MATLAB (R2024b) using published scripts[2] with following modifications. In Step1.m, Barcode 1 (GGAGAC) was used. In Step2.m, `batmax = 0; q = 18; pepstart = 'ATGTGC'; adaptor = 'TATCCTTATGATGTTCCCGATTAT'`; In Translate.m, `AmberQ = setfield(GeneticCode,'TAG','*')`.

SMILES parsing and *in silico* cyclization

The redundant amino acid sequences were removed, and their corresponding read counts were aggregated using Microsoft Excel pivot table. Sequences containing nonsense mutations (internal stop codons) or frameshift mutations resulting in loss of the HA tag were excluded from the analysis. The linker and HA tag sequences were then separated from the full-length translated peptides, generating the core MCX_nC sequences. The one-letter code amino acid sequences were then converted to three-letter codes sequences and subsequently converted to SMILES using ChemDraw (20.1.1) and ChemOffice (23.1.1.3). The resulting spreadsheet containing MCX_nC sequences, read counts, and SMILES strings for linear peptides was then used as the input for *in silico* cyclization in DataWarrior (06.01.00). Cyclization was performed using the "Enumerate Combinatorial Libraries" function with the respective linkers.

Molecular fingerprint analysis

Fingerprint analysis was performed in JupyterLab (4.3.6) using Anaconda (Anaconda Software Distribution, 24.11.2) running Python (3.12.7), with a spreadsheet format containing SMILES representations of cyclic peptides. The environment included the following key packages: NumPy (2.2.0), pandas (2.2.3), RDKit (2024.9.6), and scikit-learn (1.7). Peptide sequences were represented as matrices of Extended-Connectivity Fingerprints[3] (ECFP4; 2048-bit), which were computed using RDKit's `MorganGenerator` with the following parameters: `radius = 2` and `fpSize = 2048`. To visualize the sequence convergence, the fingerprints of the cyclic peptide libraries were then projected into two dimensional t-distributed stochastic neighbor embedding (t-SNE)[4] as implemented in

`sklearn.manifold`, using the following parameters: `n_components=2, perplexity =30, metric="jaccard"`, and `random state=42`.

Binding affinity measurement

SNCA IRE or base-paired control RNA, labeled with fluorescein (FITC) on 5' end was folded in 1X HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.2) by heating at 95 °C for 3 min, followed by snap cooling on ice for 15 minutes. Peptide stock solutions were serially diluted to desired concentrations. The folded RNA was then added into the diluted peptide solution to give a final RNA concentration of 10 nM, with the final DMSO amount was 1%. For the binding measurement of linear peptide and RNA, 200 μM TCEP was added to the peptide solution to reduce disulfide bond formation. The RNA and peptide mixtures were incubated for 15 minutes at room temperature in the dark before being transferred into a well of black 384-well plate (Greiner, 784900). The fluorescence polarization was measured using a Synergy Neo2 plate reader (1608111A) at 485/528 nm (excitation/emission). Polarization was calculated based on the parallel (I_{ll}) and perpendicular intensities ($I_{\perp}$) using **equation 1**:

$$P = \frac{I_{ll} - I_{\perp}}{I_{ll} + I_{\perp}}$$

The fluorescence polarization as a function of peptide concentration was fit to **equation 2** to calculate the equilibrium dissociate constant (K_D):

$$Y = P_{min} + \frac{P_{max} - P_{\min}}{1 + (Kd/X)^{HillSlope}}$$

where Y is the calculated polarization (*mP*); P_{max} and P_{min} are minimum and maximum polarization among all data points on the curve, respectively; *X* is the concentration of peptides.

Gel shift assay

SNCA IRE or base-paired control RNA, labeled with fluorescein (FITC) on 5' end, was folded in 1X HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.5) by heating at 95 °C for 3 minutes followed by snap-cooling on ice for 15 minutes. The peptide stock was serially diluted to 50 μM. The folded RNA was then added into the diluted peptide solution to give a final concentration of 1 μM, with the final DMSO concentration was 1%. The RNA and peptide mixtures were incubated for different time points at room temperature in the dark. The reactions were quenched by addition of 2X RNA loading dye and stored at -20 °C or immediately loaded on a gel. Samples were resolved on a denaturing 15% PAGE gel at 200V (pre-run for 15 minutes). The fluorescence was detected at 492/508 nm (excitation/emission).

To calculate rate constant for RNA labeling with FS-2, folded *SNCA* IRE (2.5 μM) was incubated with FS-2 at a final concentration of 100, 50, 25 and 12.5 μM at 25 °C. Sample aliquots were quenched at different time point with 2X RNA loading dye and store at -20 °C. After collecting all samples, a denaturing gel was used to resolve the samples and shifted bands were quantified to calculate the percentage of labeled RNA at each time point. A first-order association equation (**equation 3**) was used to calculate the observed rate constant for each FS-2 concentration:

$$Y = 100 * (1 - e^{-k_{obs} * t})$$

Where *Y* represents the percentage of shifted RNA, *T* is time in minutes, k_{obs} is the rate constant with unit in inverse minutes. k_{obs} values for each concentration of FS-2 were then fit to **equation 4** to determine the kinact and K_I.

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]}$$

Where [I] corresponds to the concentration of FS-2 in M. Reported k_{inact} and K_I are average of two independent experiments.

Peptide stability test

Peptides were dissolved in 1X HEPES buffer at a final concentration of 200 μM in a total volume of 100 μL solution. At each time point, 15 μL of the reaction mixture was aliquoted and quenched with 15 μL of LC-MS grade water containing 0.5% formic acid. A 5 μL of this mixture was immediately injected into the LC-MS.

Sample analysis was performed on a Agilent 6130 LCMS equipped with an ESI/APCI multimode ion source and a diode array detector (DAD). Separation was achieved on a 3 × 50 mm reversed-phase Agilent Poroshell 120 EC-C18 (2.7 μm) analytical column at 25 °C using the following solvent system: solvent A, LCMS grade water with 0.1% formic acid, solvent B, LCMS grade acetonitrile with 0.1% formic acid at a flow rate of 1 mL/min. The gradient was as follows: 5% B→50% B over 6 min, 50% B→ 95% B over 3 min, 95% B for 1 min, 95% B→5% B over 0.2 min followed by 5% B over 3 min. Elution profiles were recorded at four wavelength at 214, 254, 260 and 280 nm with a reference wavelength at 360 nm.

SHAPE analysis

The SHAPE experiment was performed according to previously published protocols [5,6]. RNA was transcribed from a PCR amplified DNA template using MEGAscript T7 Transcription Kit following the manufacturer's protocol. Primers used were listed in Table S2. The transcribed RNA was purified with denaturing 10% polyacrylamide gel and recovered by ethanol precipitation. The transcription template sequence is as follows:
TAATACGACTCACTATAGGCGCTGGGAGTGGCCATTCGACGACAGTGTGGTGTAAAGGAATTC
ATTAGCGCCGTTGAAAGATGAGCCG (NED-tagged reverse transcription primer landing region is underlined).

For each sample used in SHAPE experiment, 5 pmol of RNA was folded in HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.2) by heating at 95 °C for 3 minutes followed by cooling on ice for 3 minutes. Next, a 3X folding buffer (300 mM HEPES, 20 mM $MgCl_2$, 300 mM NaCl, pH 8.0) was added, followed by incubation with peptide (100X stock solution) or DMSO at the desired concentration. The samples were incubated at room temperature for 30 minutes for ***m*-DBX** cyclized peptides or 1 h for **DBX-FS** cyclized peptides. Then, 2 μL of NMIA stock solution (130 mM in DMSO) was added, and the reaction was incubated at 37 °C 45 minutes. For RT stop experiment, folded RNA was incubated with FS-2 at varying concentrations at 37 °C for 24 hours in buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.5).

The reaction was stopped by adding 10 volumes of precipitation mixture (0.3 M NaOAc, 0.2 mg/mL of glycogen and ethanol). RNA was precipitated overnight at -80 °C, followed by centrifugation for 30 minutes at 4 °C. The pellet was washed with 80% cold ethanol and re-centrifuged. After removal of supernatant, the pellet was air dried and resuspended in nuclease-free water.

For the primer extension step, purified RNA was mixed with 0.6 µL of a 10 pmol/µL NED-labeled RT primer, 0.25 µL of a 10 mM dNTP mix, and nuclease-free water to a final volume of 6.25 µL. The mixture was heated at 75 °C for 5 minutes followed by cooling on ice. Next, 2 µL of 5X first strand buffer, 1 µL of 0.1 M DTT, 0.5 µL of SUPERase·In RNase Inhibitor and 0.25 µL of SuperScript III reverse transcriptase were added. The reaction was incubated sequentially at 25 °C for 10 minutes, 50 °C for 50 minutes, and 55 °C for 50 minutes. Upon completion, 1 µL of 4 M NaOH was added to each sample and the mixture was heated at 95 °C for 3 min to degrade the RNA. Subsequently, 4 µL of 1 M Tris buffer (pH 7.5) was added, and the samples were mixed with 2× loading dye and heated again at 95 °C for 3 min. The samples were then loaded on a denaturing 10% polyacrylamide gel (16.5 cm × 28 cm) and resolved at constant 450 V for 3 hours. Gel images were acquired using a fluorescence imager (Typhoon, GE Healthcare) in the Cy3 channel (554/568 nm, excitation/emission). For each sequencing experiment, 1 µL of ddATP and ddCTP were included to generate termination product ladders corresponding to U and G, respectively.

RNA degradation assay

SNCA IRE or control RNA labeled with fluorescein (FITC) at the 5' end was folded in 1X HEPES buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.5) by heating at 95 °C for 3 min followed by snap cooling on ice for 15 minutes. Peptide stock solutions were serially diluted to 50 µM. Folded RNA was supplemented with BSA (40 µg/mL) and added into the diluted peptide solution to give a final concentration of 1 µM so the final concentration of DMSO was 1%. The RNA and peptide mixtures were incubated at 37 °C for various time points. Then the reactions were quenched by the addition of 2X RNA loading dye, and the samples were stored at -20 °C or immediately loaded on a denaturing 15% PAGE gel (constant at 200V). The gels were imaged for FITC signals at 492/508 nm (excitation/emission).

For the validation of RNA degradation sites with large gel (16.5 cm × 28 cm). Samples were loaded on denaturing 15% polyacrylamide gel and resolved at constant 450 V for 4 hours. Hydrolysis ladder and T1 ladder were prepared according to published procedures[7]. Briefly, 3 µL of 100 µM FITC labeled RNA were heated at 95 °C for 5 minutes in hydrolysis buffer (50 mM sodium bicarbonate, 1 mM EDTA, pH 9.4). For T1 digested lanes, 3 µL of 100 µM FITC labeled RNA were heated at 60 °C for 10 minutes in RNase T1 buffer (20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea) followed by cooling to room temperature. Then 1.5 µL of RNase T1 (100 U/µL or 10 U/µL) were added into the solution and further incubated at room temperature for 20 minutes. All ladders were then mixed with 2X RNA loading dye before being loaded on the gel.

Enzymatic digestion and mass spectrometric analysis of FS-2 modified RNA

100 µg of purified *SNCA* IRE was folded in 990 µL of 1X HEPES buffer (pH 7.5) by heating at 95 °C for 3 minutes and snap-cooled on ice for 15 minutes. Then the solution was supplemented with 10 µL of 10 mM FS-2 (to reach a final concentration of 100 µM) at a peptide-to-RNA ratio of 20:1 and incubated at 25 °C for 24 hours. Upon completion, the RNA sample was purified by ethanol precipitation. Briefly, 0.1

volume of 3 M sodium acetate, 2.5 volumes of cold ethanol and 2.5 µL of glycogen (20 mg/mL) were added, and the sample was incubated at -70 °C overnight. RNA was pelleted by centrifugation at 15000 G for 15 minutes at 4 °C followed by washing with 80% cold ethanol, and air-dried at room temperature with the tube left open until all solvent was evaporated. Then the sample was resuspended with 100 µL of nuclease-free water and quantified with a nanodrop spectrophotometer.

For enzymatic digestions, 25 µg of the labeled RNA sample was digested in a 100 µL reaction using a nucleoside digestion mix. The reaction included 10 µL of 10X reaction buffer, 5 µL of enzyme mix and was incubated at 37 °C overnight.

Digested samples (5 µg) were acidified to 0.5% formic acid prior to injection and separation on an Agilent 6540 Q-TOF LC-MS equipped with a Jet Stream Technology Ion Source (AJS) and a Diode Array Detector (DAD). Eluent nebulization was achieved with a N_2 pressure of 35 (psi), and solvent evaporation was assisted by a flow of N_2 drying gas (300 °C). Mass spectra were collected in positive-ion mode with a capillary voltage of 3.5 kV and fragmentor at 170 V over a 20-2800 *m/z* scan range. Precursor ions were identified in HPLC traces after separation of injected samples on a 3 × 50 mm reversed-phase Agilent Poroshell 120 EC-C18 (2.7 µm) analytical column at 25 °C using the following solvent system: solvent A, LCMS grade water with 0.1% formic acid, solvent B, LCMS grade acetonitrile with 0.1% formic acid at a flow rate of 0.6 mL/min; 5% B for 5 min, 5% B→35% B over 30 min, 35% B →95% B over 4 min, 95% B for 1 min, 95% B→5% B over 0.2 min followed by 5% B over 3 min. Elution profiles were recorded at four wavelength at 214, 254 , 260 and 280 nm with a reference wavelength at 360 nm. Fragmentation of selected ions in MS/MS experiments was achieved by collision induced dissociation (CID) using targeted MS/MS function. Targeted ions identified from previous experiments were fragmented with 30 or 40 eV (medium isolation width, delta retention time is two minutes). Data analysis was performed using Agilent BioConfirm Software (12.1) and MestReNova (14.2.0-26256).

RNA folding predictions and dockings with cyclic peptides

The sequence below was used to predict the 3D structure of *SNCA* IRE:

5'– GCGCUGGGAGUGGCCAUUCGACGACAGUGUGGUGUAAAGGAAUUCAUUAGCGC – 3'

Three extra nucleotides were appended to each end to form closed base-pairs. The SMILES input file for FS-2 was generated using DataWarrior (v06.04.01). RNA prediction and docking was performed by Boltz-1[8,9] (https://neurosnap.ai/). Diffusion Samples were set to 1-5. Number Recycles were 10. Sampling Steps were 200. RNA and cyclic peptide interactions were analyzed and visualized using UCSF ChimeraX (1.9).

Solid phase peptide synthesis

Peptides were synthesized using solid-phase peptide synthesis (SPPS) and Rink Amide resin at a scale of at least 0.05 mmol. Briefly, Fmoc deprotection was carried out using 20% piperidine in dimethylformamide (DMF) for three times (4 minutes each time for peptides shorter than 10 amino acids and 7 minutes for longer peptides). Amide coupling was performed by pre-activating N-terminal Fmoc-protected amino acids (4 eq.), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-

oxide hexafluorophosphate, (HATU, 4 eq.), 1-Hydroxy-7-azabenzotriazole (HOAT, 4 eq.) and *N*,*N*-Diisopropylethylamine (DIPEA, 8 eq.) in DMF for 15 minutes prior to mixing with resin. Coupling reactions were carried out at room temperature for 2 hours for most amino acids except for proline and arginine, which were coupled for 3 hours. After each coupling and final deprotection step, the resin was washed three times with DMF, three times with dichloromethane (DCM) and six times with DMF. Following the final amino acid coupling step, the resin was washed with DMF (four times) and DCM (five times), then dried under reduced pressure before storage at -20 °C.

N-terminal modification with compound **4**

Compound **4** (4 eq.) was deprotected in 5 mL of 50% trifluoroacetic acid (TFA) in DCM for 1 hour at room temperature, with reaction progress monitored by LC-MS. Upon completion of deprotection, the solvent was removed by rotavap, and the remaining product was dried under vacuum overnight. The crude product was then mixed with HATU (4 eq.), HOAT (4 eq.) and DIPEA (8 eq.) in DMF and activated for 15 minutes before being added to the Fmoc-deprotected peptide. The reaction mixture was agitated at room temperature for 3 hours.

Peptide cleavage and deprotection

A portion of the resin was weighted and subjected to Fmoc deprotection. Following the washing steps, the resin was incubated in a cleavage cocktail solution consisting of TFA/water/phenol/thioanisole/1,2-ethanedithiol (EDT) (82.5:5:5:5:2.5) for 4-6 hours. The cleavage mixture was then precipitated by dropwise addition into ice-cold diethyl ether. A suspension usually was observed at this step. This solution was stored at -20 °C until most of the white precipitate had formed. The precipitate was collected by centrifugation at 5000 × G for 10 minutes at 4 °C, and the supernatant was discarded. The resulting pellet was washed with additional cold ether and then resuspended in a mixture of H_2O/acetonitrile/TFA (50:50:0.1). The samples were lyophilized overnight.

Peptide purification

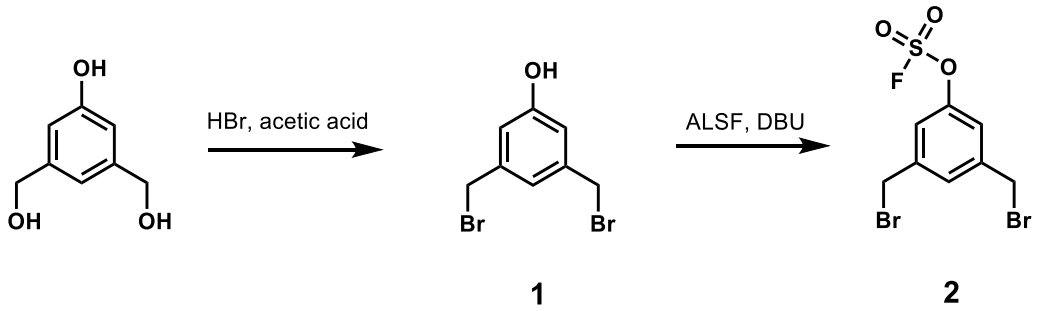
The lyophilized peptide was redissolved in minimum amount of acetonitrile and water, with the solvent ratio adjusted based on peptide solubility. The solution was titrated with TFA to achieve an acidic pH and filtered through a 0.2 μm PVDF filter before purification by reverse-phase high-performance liquid chromatography (HPLC) (ACCQPrep HP150). Purification was performed on a preparative reverse-phase HPLC column (Agilent Zorbax Eclipse Plus C18, 212 × 250 mm, 5 μm using the following solvent system: solvent A, water/ 0.1% trifluoroacetic acid (TFA), and solvent B, acetonitrile/0.1% TFA, at a flow rate of 5 mL/min and a gradient of 5% B for 5 min, 5% B→50% B over 35 min, 50% B→100% B over 5 min, 100% B for 5 min followed by 100% B→5% B over 5 min. The initial acetonitrile content was adjusted based on peptide solubility. Elution profiles were monitored at 214 nm and 280 nm, with fractions collected at 214 nm. The desired fractions were collected and lyophilized to obtain the final purified product as a white powder. The resulting product was either dissolved in anhydrous DMSO or store at powder at -20 °C.

Cyclization of hit peptides with linkers

A total scale of 10 mg of purified peptide and tris(2-carboxyethyl)phosphine (TCEP) (1.2 eq.) were dissolved in 4 mL of a degassed solvent mixture containing 40% acetonitrile in water. To this solution, 0.5 mL of 1 M ammonium bicarbonate solution (above pH 7.8) was added, and the mixture was incubated at room temperature for 10 minutes. Subsequently, ***m*-DBX** or compound **2** (1.4 eq.), pre-dissolved in 0.5

mL of acetonitrile, was added to the reaction mixture and the reaction was stirred for one hour. The reaction progress was monitored by ESI-LC-MS. Upon completion, the reaction was quenched with 50 µL of TFA and lyophilized. The crude product was analyzed by ESI-LC-MS and purified by preparative HPLC following the methods mentioned above.

Synthesis of dibromoxylene derivatives and imidazole tag

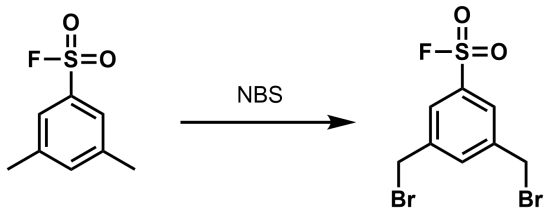


Scheme S1. Synthesis of compound **1** and **2**.

Synthesis of 3,5-bis(bromomethyl)phenol (1)

The synthesis of compound **1** was modified from published protocol[10]. 3,5-Di(hydroxymethyl)phenol (500 mg, 3.2 mmol) was dissolved in acetic acid (4 mL) and cooled down to 0 °C. Subsequently, 1.6 mL of 33% HBr in acetic acid was added dropwise to solution, and the solution was stirred at room temperature for 24 hours. The reaction progress was monitored with thin-layer chromatography (TLC) using hexane: ethyl acetate (3:1). Upon completion, 20 mL of distilled water was added, and the solution was stirred for an additional 10 minutes. The reaction mixture was then extracted three times with CH_2Cl_2. The combined organic layers were washed sequentially with distilled water, saturated $NaHCO_3$, and brine. The crude product was further purified using flash chromatography with ethyl acetate/hexane mixture (0-15%). Yield: 28%. ^{1}H NMR (400 MHz, $CDCl_3$) δ6.99 (s, 1H), 6.81 (d, J= 1.5 Hz, 2H), 5.01 (s, 1H), 4.40 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ155.82, 139.96, 122.12, 116.17, 32.56.

Synthesis of 3,5-bis(bromomethyl)phenyl sulfurofluoridate (2) Compound **1** (170 mg, 0.61 mmol) and 1.2 equivalent 4-(Acetylamino)phenyl]imidodisulfuryl difluoride (ALSF) (230 mg, 0.73 mmol) were dissolved in anhydrous DCM. 1 equivalent of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was added in two portions sequentially with 30-minutes interval between additions. The reaction mixture was stirred at room temperature for 1 hour, with progress monitored by TLC using 50% ethyl acetate in hexane. Upon completion, the reaction mixture was diluted with DCM, washed with brine, and the organic layers were concentrated and purified with silica gel flash chromatography with a gradient of 0-60% of ethyl acetate in hexane. Yield: 40%. ^{1}H NMR (400 MHz, $CDCl_3$) δ7.47 (s, 1H), 7.32 (s, 2H), 4.46 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ149.88, 141.23, 129.63, 121.29, 30.78. ^{19}F NMR (376 MHz, $CDCl_3$) δ38.40. HR-MS (ESI, positive-ion mode): m/z calculated for $C_8H_7Br_2FO_3S$, 359.8467; found, 359.8464.

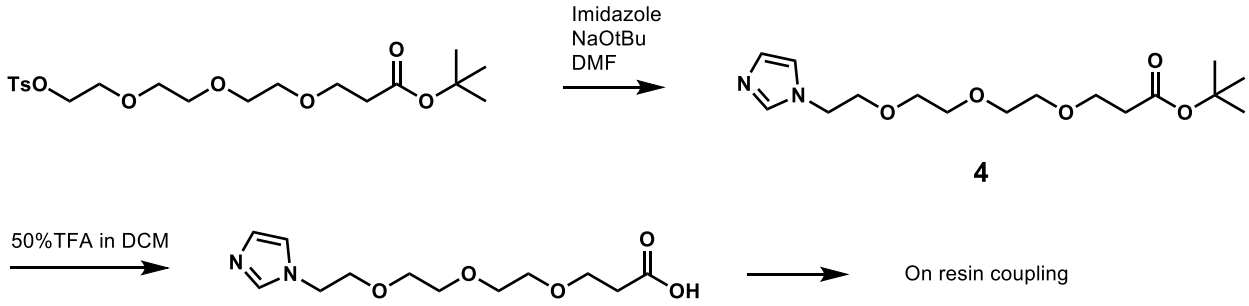


3

Scheme S2. Synthesis of compound **3**

Synthesis of 3,5-bis(bromomethyl)benzenesulfonyl fluoride (3)

3,5-Dimethylbenzenesulfonyl fluoride (200 mg, 1.0 mmol) was dissolved in 5 mL of acetonitrile and supplemented with and 2.2 equivalent of *N*-bromosuccinimide (NBS) (416.5 mg, 2.2 mmol) and 0.01 equivalent of azobisisobutyronitrile (1.7 mg, 0.01 mmol). The reaction was heated under reflux overnight. Upon completion, the solvent was removed by rotary evaporator, and the remaining residue was purified by flash chromatography using 0-60% of ethyl acetate in hexane. Yield: 8.7%, ^{1}H NMR (400 MHz, $CDCl_3$) δ7.96 (d, 2H), 7.82 (s, 1H), 4.52 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ140.84, 136.38, 134.36, 134.11, 128.37, 30.21. ^{19}F NMR (376 MHz, $CDCl_3$) δ66.12. HR-MS (ESI, positive-ion mode): m/z calculated for $C_8H_7Br_2FO_2S$, 343.8518; found, 343.8415.



4

Scheme S3. Synthesis of compound **4**

Synthesis of *tert*-butyl 3-(2-(2-(2-(1H-imidazol-1-yl)ethoxy)ethoxy)ethoxy)propanoate (4)

Imidazole (22 mg, 0.32 mmol) and sodium *tert*-butoxide (25.9 mg, 0.3 mmol) were mixed in anhydrous DMF (2 mL) at 0 °C in a N_2 pre-flushed round bottom flask. The mixture was kept at room temperature and stirred for 1 hour. Separately, *tert*-butyl 3-(2-(2-(2-(tosyloxy)ethoxy)ethoxy)ethoxy)propanoate (174.7 mg, 0.4 mmol) was dissolved in anhydrous DMF (3 mL) and this solution was mixed with previous solution while on ice. The mixture was stirred at room temperature for an additional hour and the reaction progress was monitored by TLC (10% methanol in DCM, with ninhydrin staining) and LC-MS. Upon completion, the product was extracted with ethyl acetate and washed sequentially with water and brine. The organic solvent was then removed under vacuum and the crude product was purified using flash chromatography (50% - 95% of ethyl acetate in hexane to 10% methanol in ethyl acetate with 0.1% ammonia). The purified product was obtained as a yellowish oil. Yield: 40%. ^{1}H NMR (400 MHz, $CDCl_3$) δ7.53 (s, 1H), 7.04 (s, 1H), 7.00 (s, 1H), 4.11 (t, 2H), 3.69 – 3.76 (m, 4H), 3.57 – 3.63 (m, 8H), 2.50 (t, 2H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ170.87, 137.41, 128.68, 119.55, 80.50, 70.67, 70.55,

70.52, 70.44, 70.34, 66.87, 47.13, 36.25, 28.07. HR-MS (ESI, positive-ion mode): m/z calculated for $C_{16}H_{28}N_2O_5$, 328.1998; found, 328.2033.

^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR characterizations (Figure S51-S60)

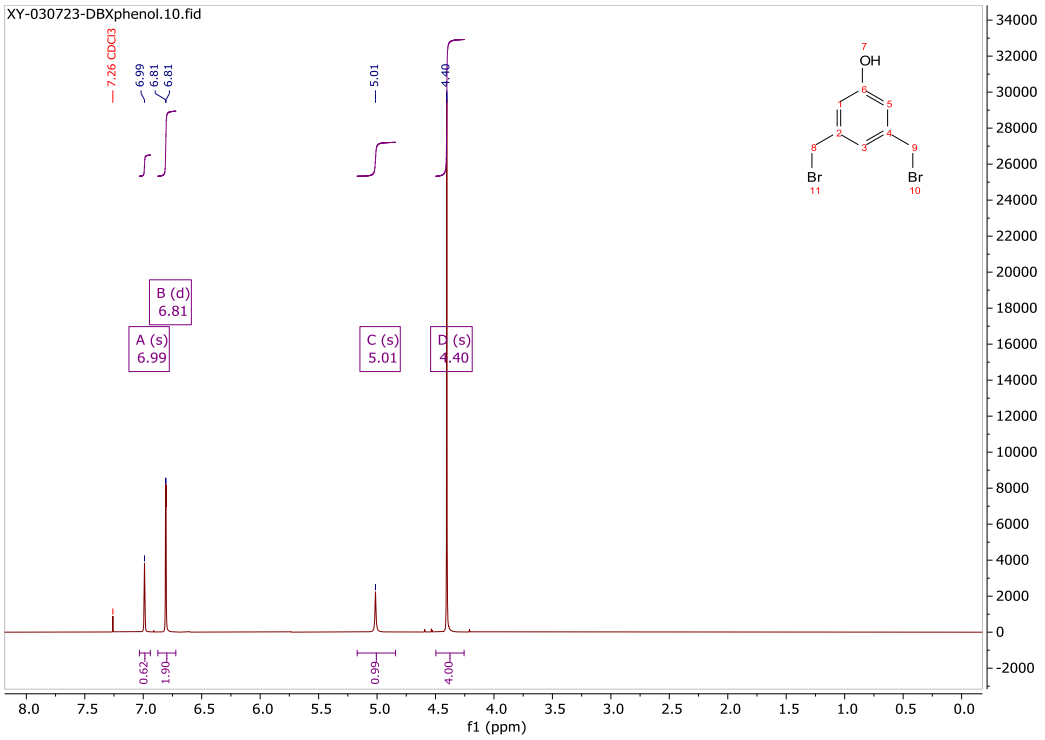


Figure S51. ^{1}H NMR spectrum of compound **1** (400 MHz, $CDCl_3$)

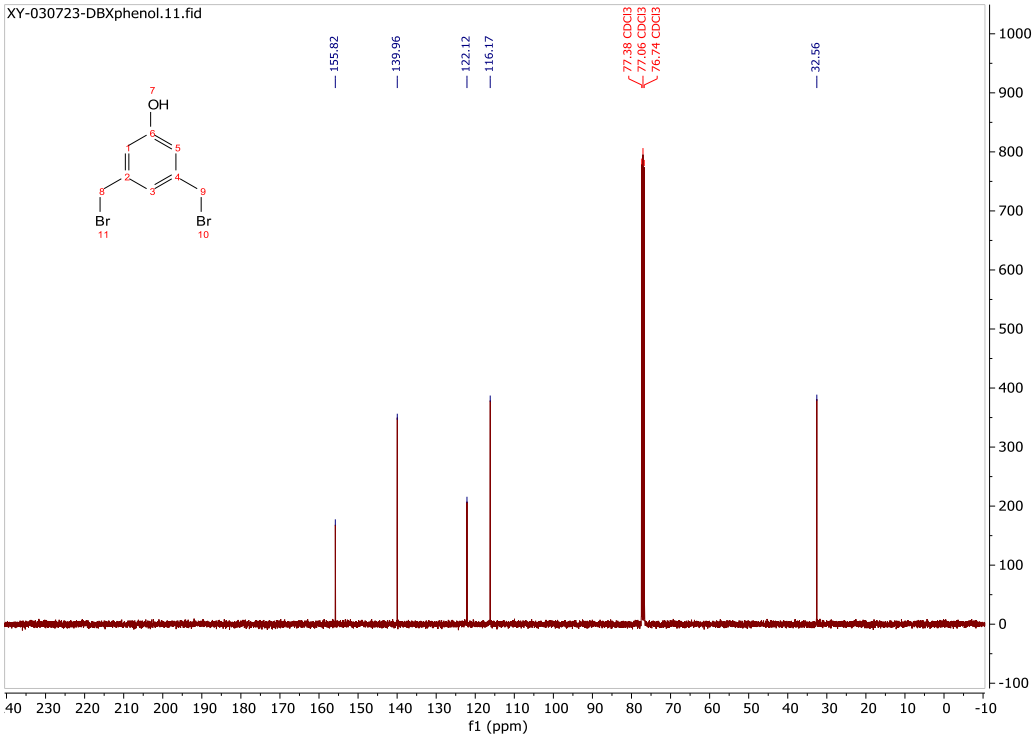


Figure S52. ^{13}C NMR spectrum of compound **1** (400 MHz, $CDCl_3$)

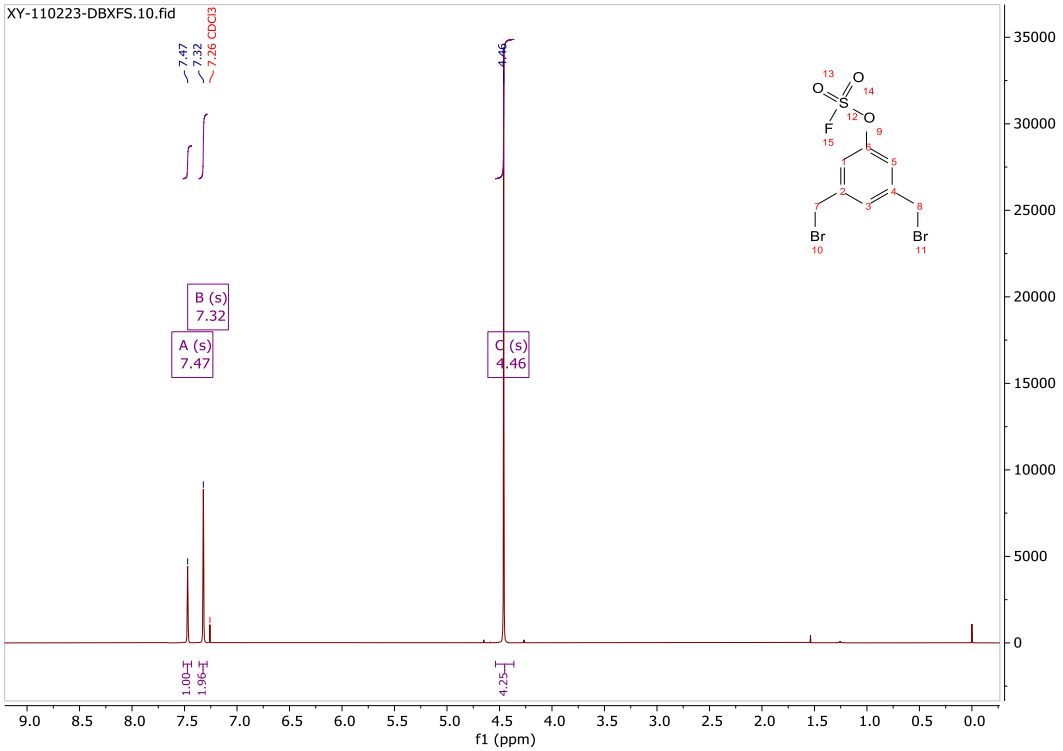


Figure S53. ^{1}H NMR spectrum of compound **2** (400 MHz, $CDCl_3$)

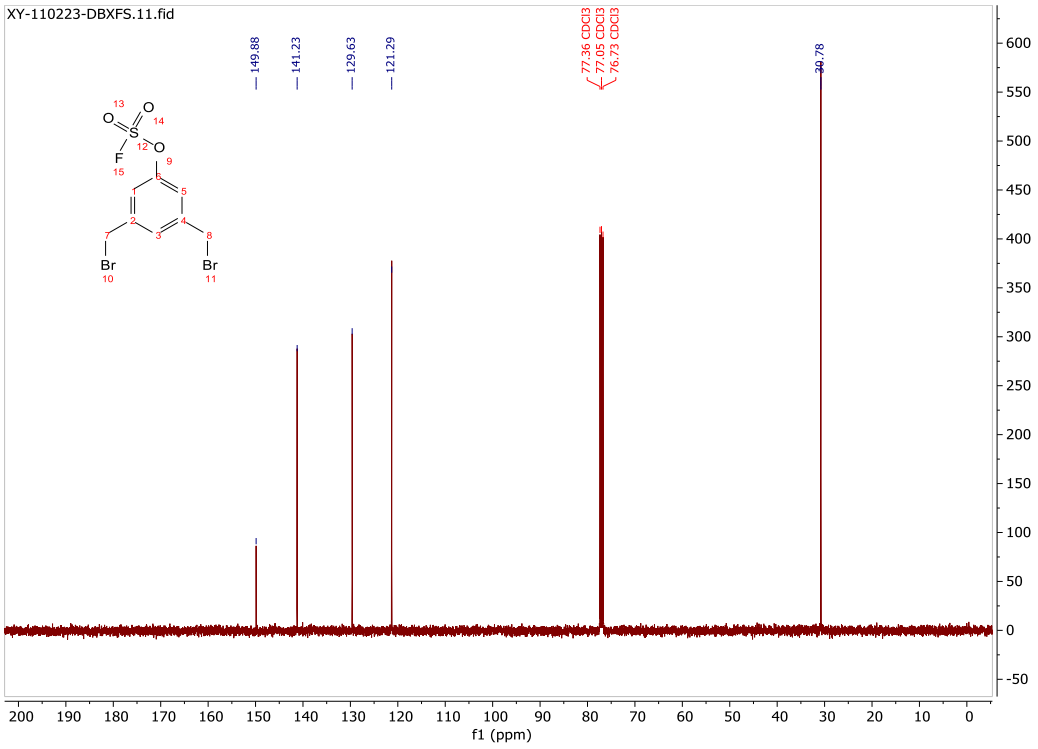


Figure S54. ^{13}C NMR spectrum of compound **2** (400 MHz, $CDCl_3$)

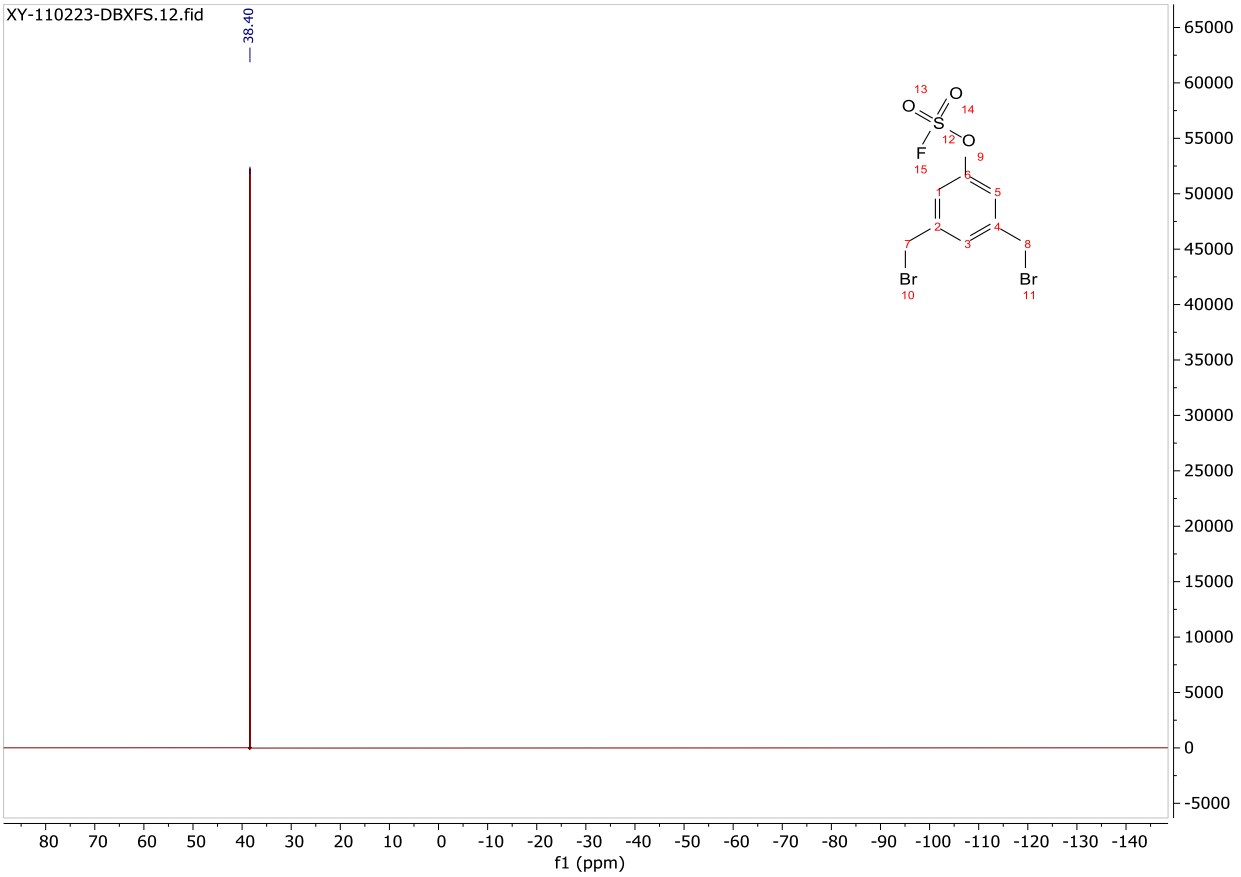


Figure S55. ^{19}F NMR spectrum of compound **2** (400 MHz, $CDCl_3$)

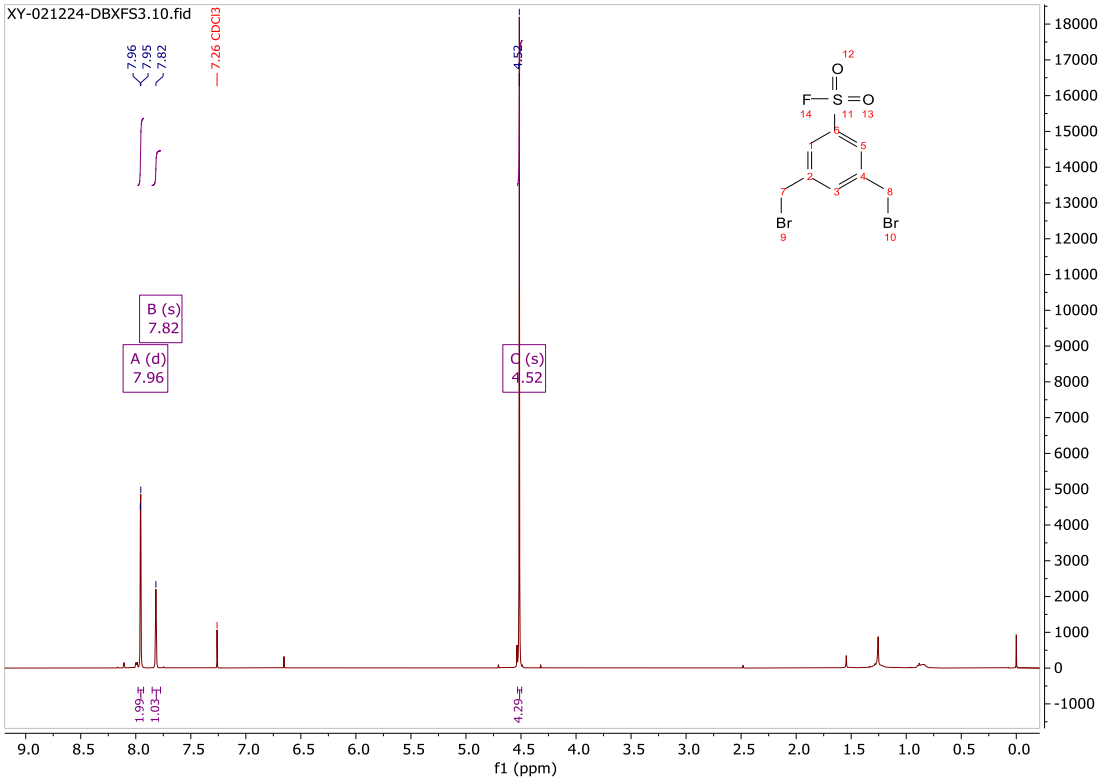


Figure S56. ^{1}H NMR spectrum of **compound 3** (400 MHz, $CDCl_3$)

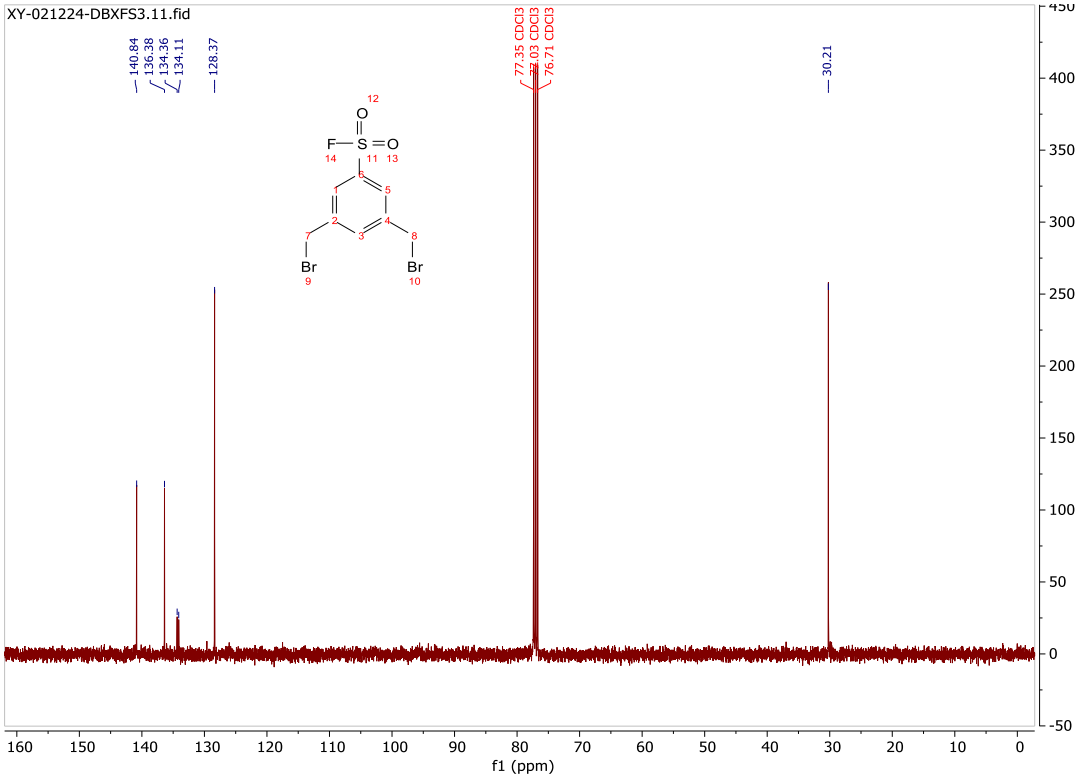


Figure S57. ^{13}C NMR spectrum of compound **3** (400 MHz, $CDCl_3$)

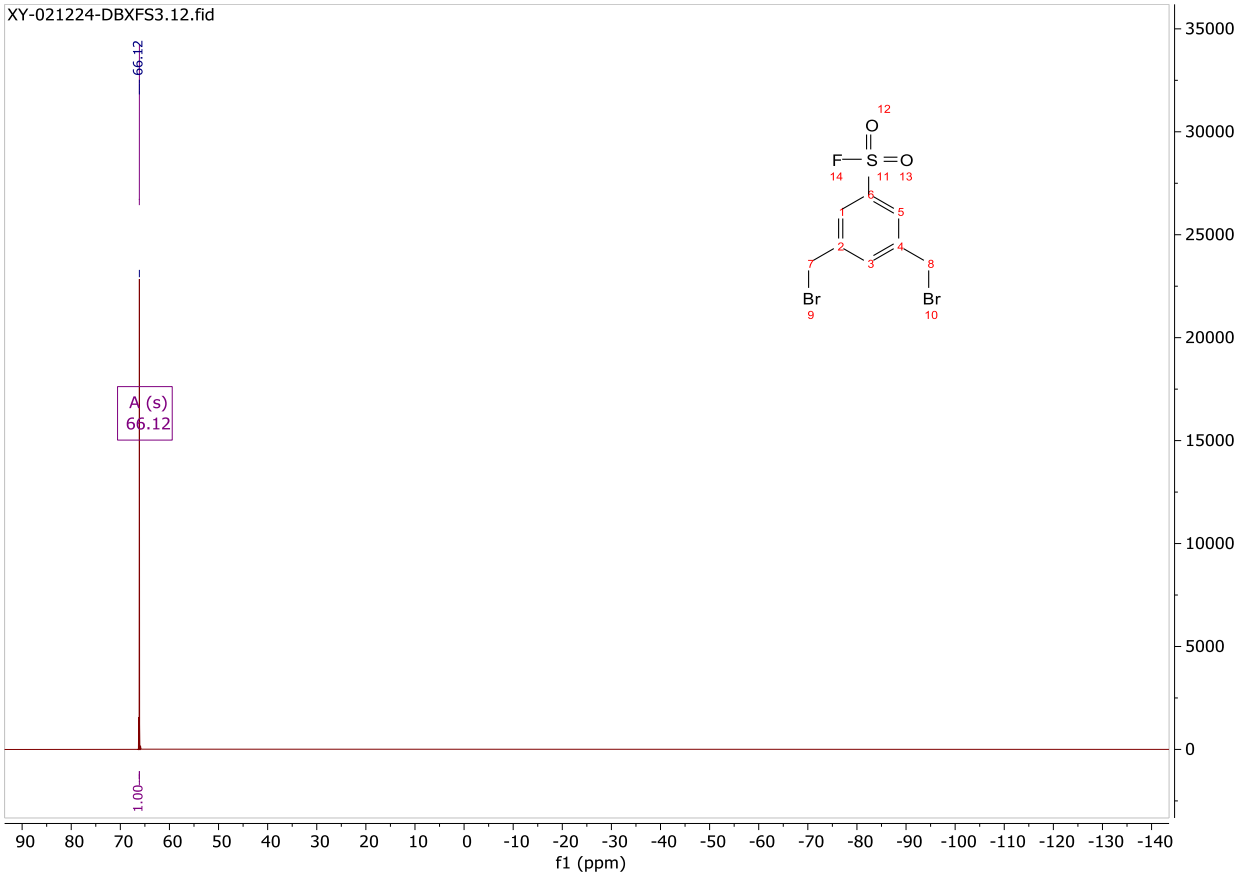


Figure S58. ^{19}F NMR spectrum of compound **3** (400 MHz, $CDCl_3$)

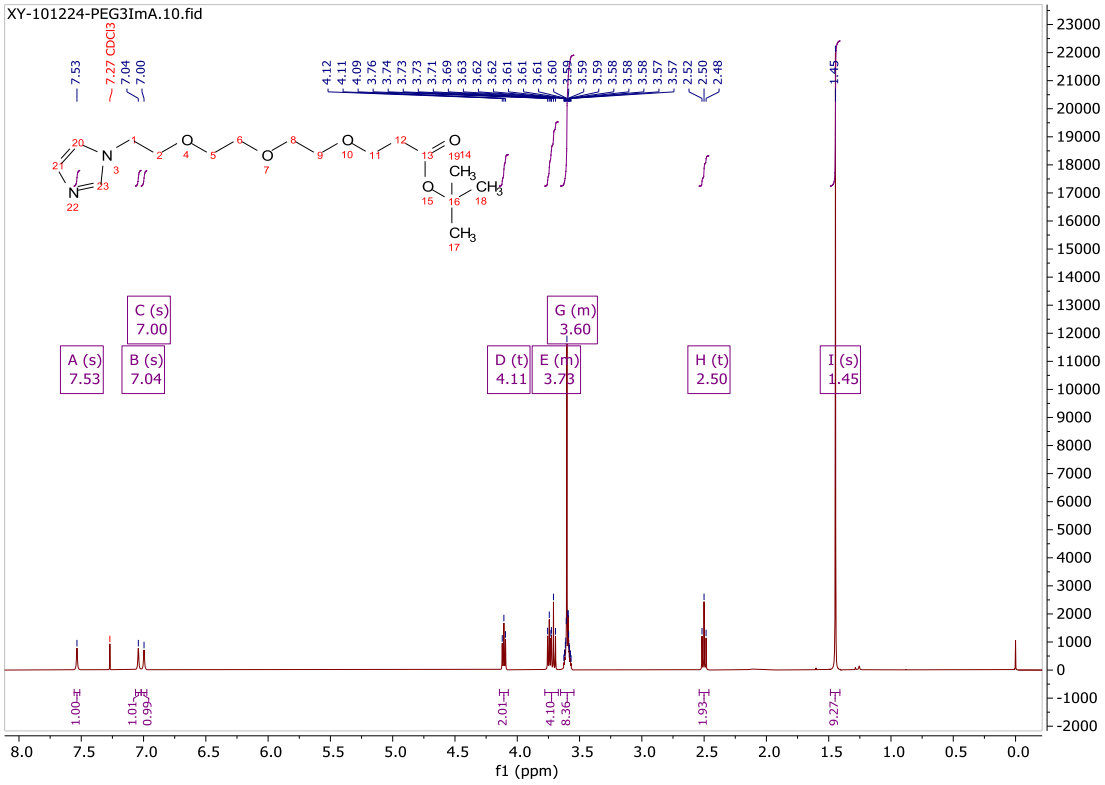


Figure S59. ^{1}H NMR spectrum of compound 4 (400 MHz, $CDCl_3$)

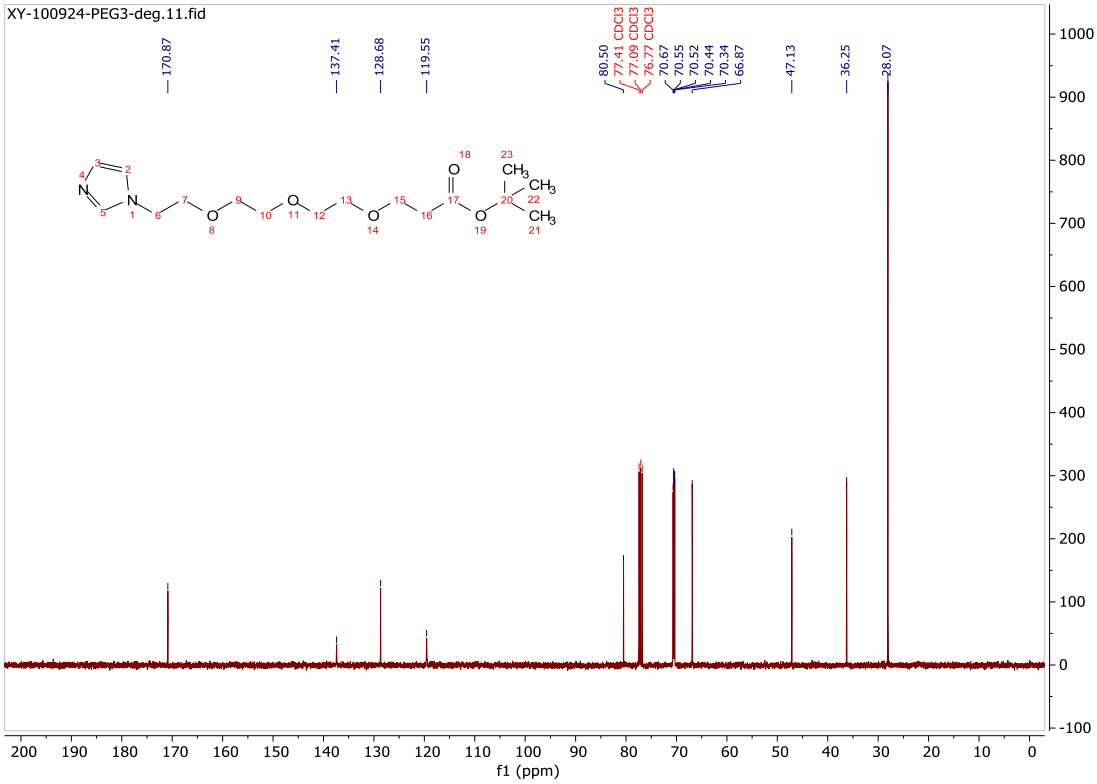
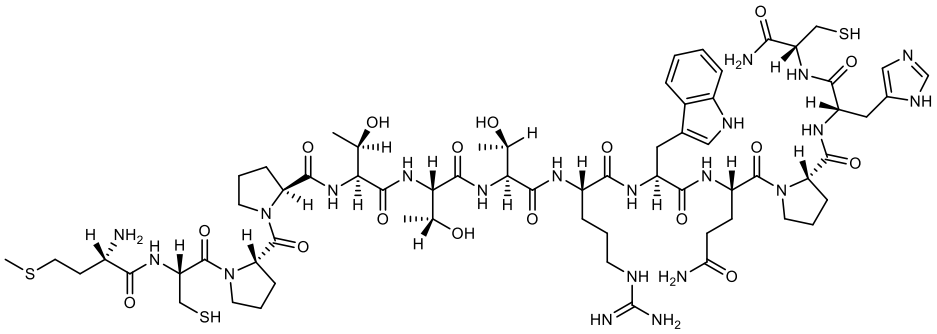


Figure S60. ^{13}C NMR spectrum of compound **4** (400 MHz, $CDCl_3$)

ESI-LC-MS analysis of linear peptides and cyclized peptides (Figure S61-S74)

Peptide: DBX-1

Sequence: MCPPTTTRWQPHC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{66}H_{101}N_{21}O_{17}S_3$, 1555.6846; found, 1555.6935.

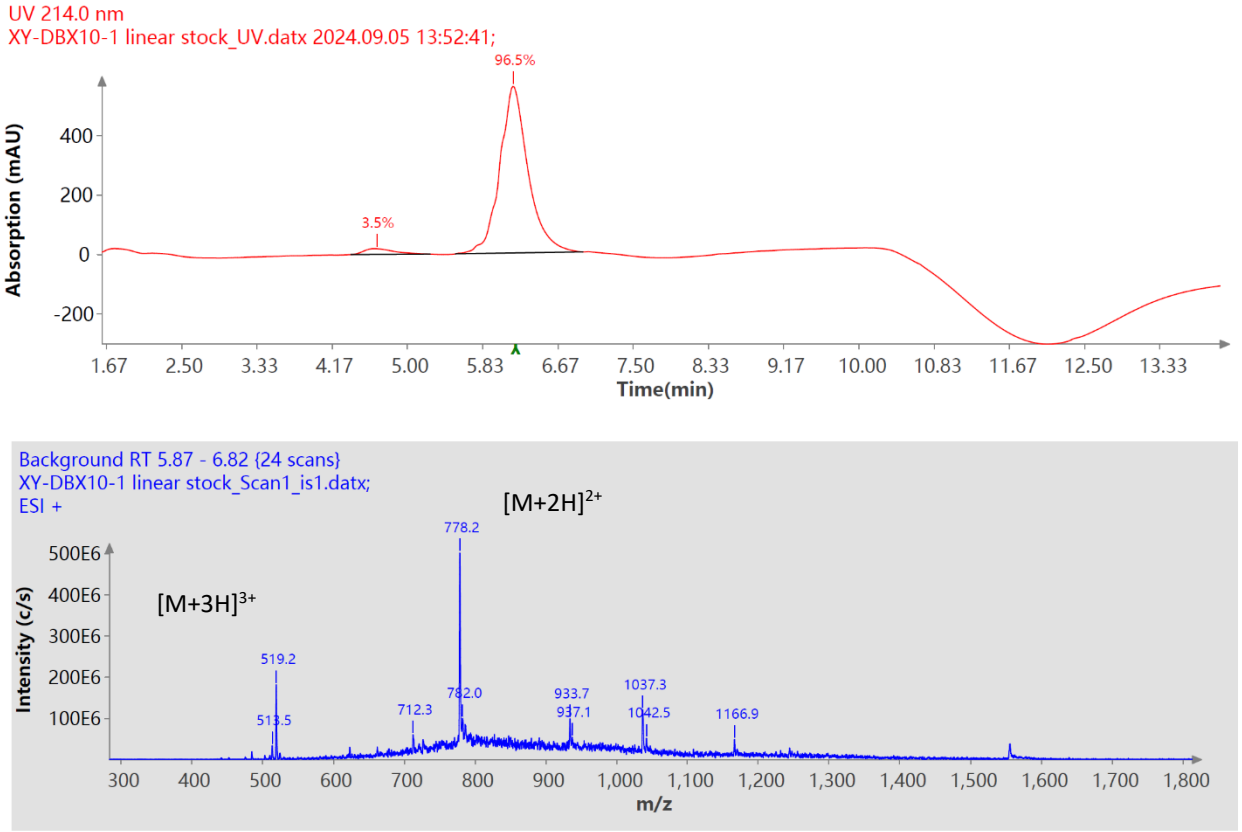
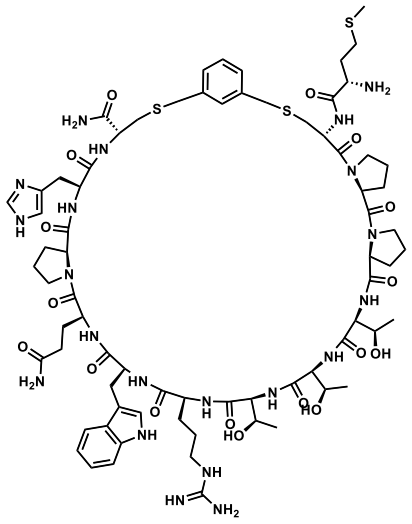


Figure S61. ESI-LC-MS characterization of linear peptide DBX-1.

Peptide: Cyclized DBX-1

Sequence: MCPPTTTRWQPHC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{74}H_{107}N_{21}O_{17}S_3$, 1657.7316; found, 1657.7358.

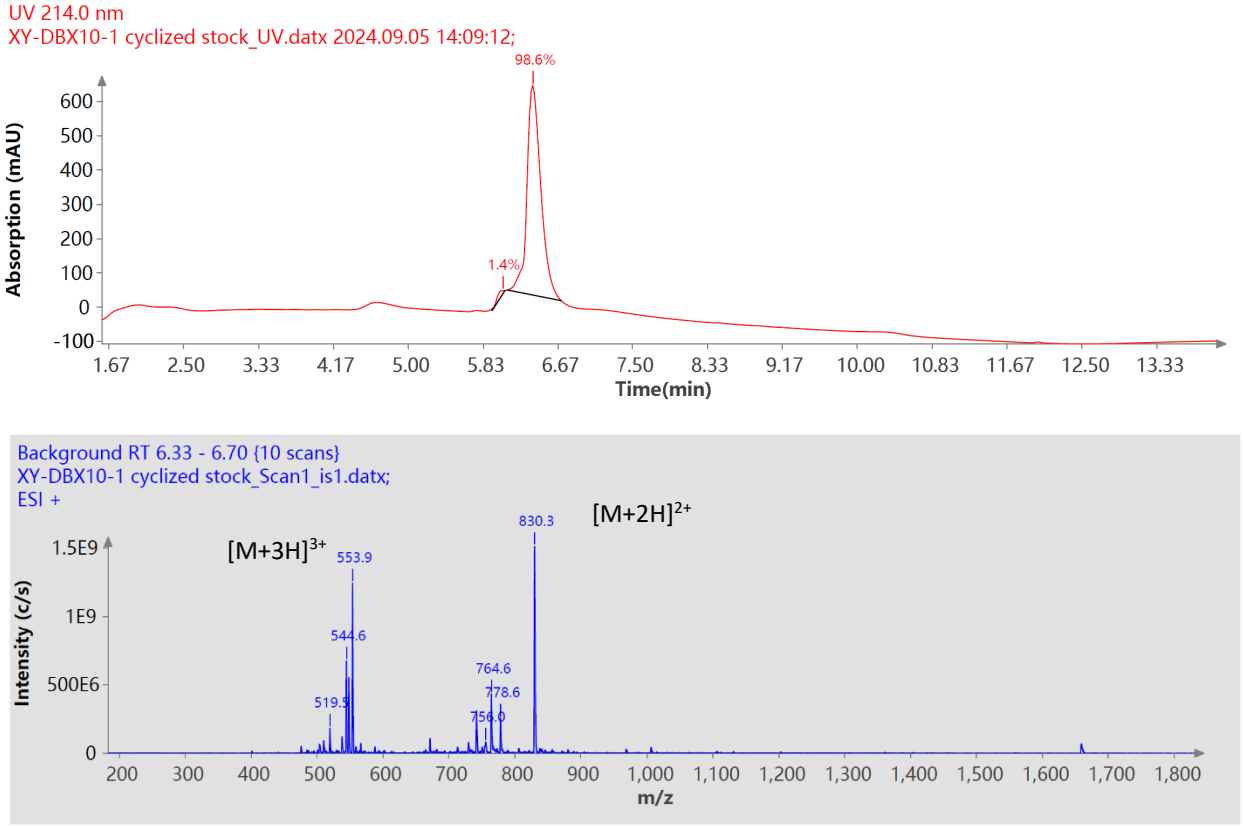
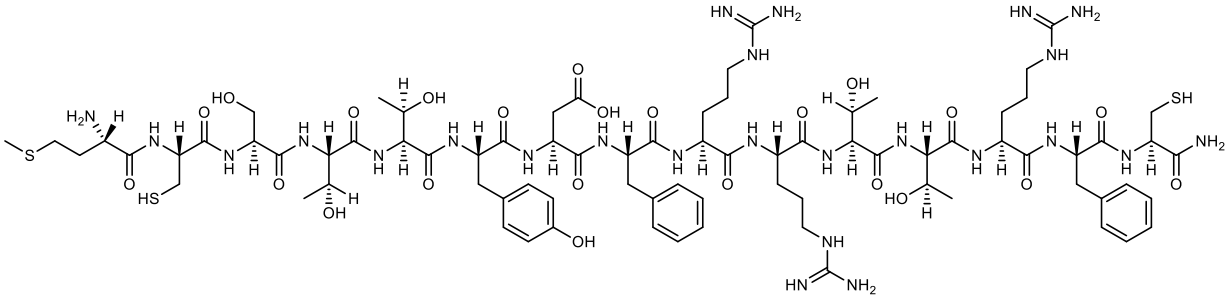


Figure S62. ESI-LC-MS characterization of cyclized peptide DBX-1.

Peptide: DBX-2

Sequence: MCSTTYDFRRTTRFC



m/z calculated for $C_{79}H_{123}N_{25}O_{23}S_3$, 1885.8386.

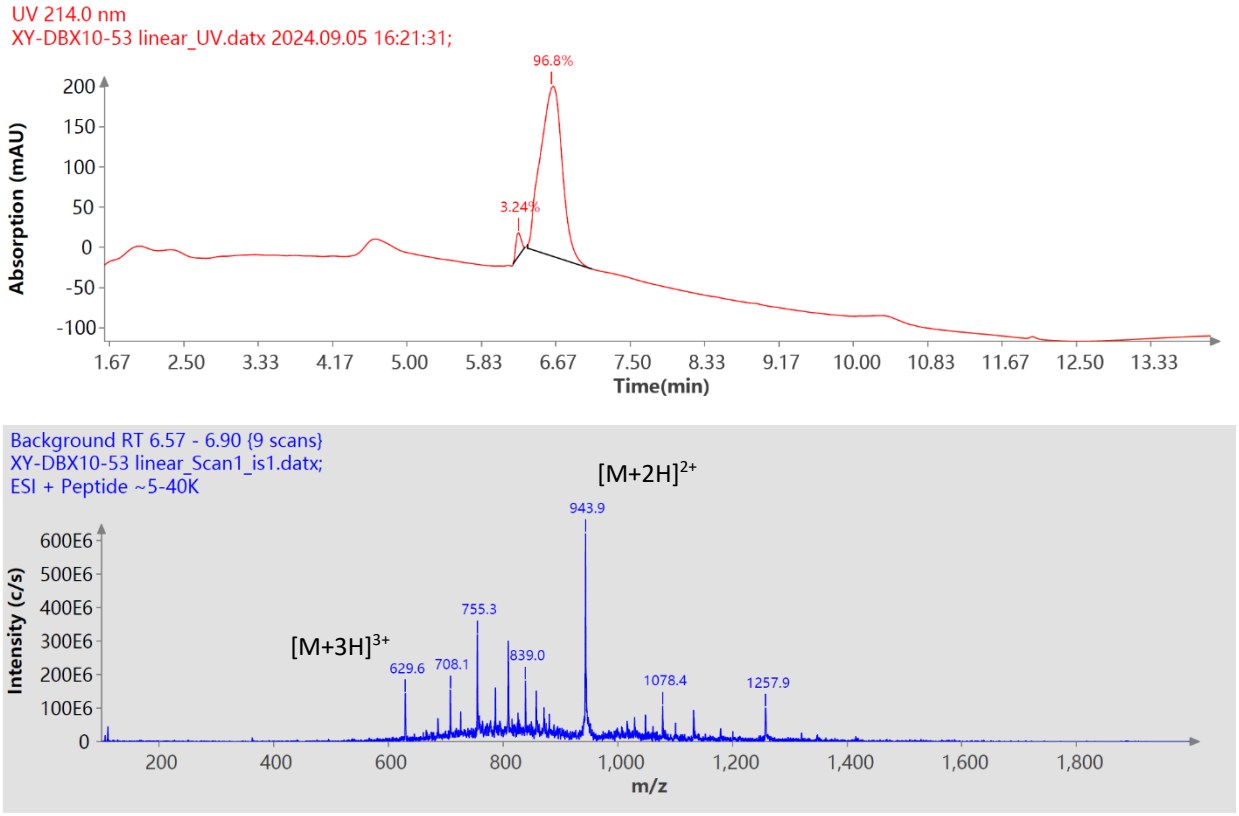
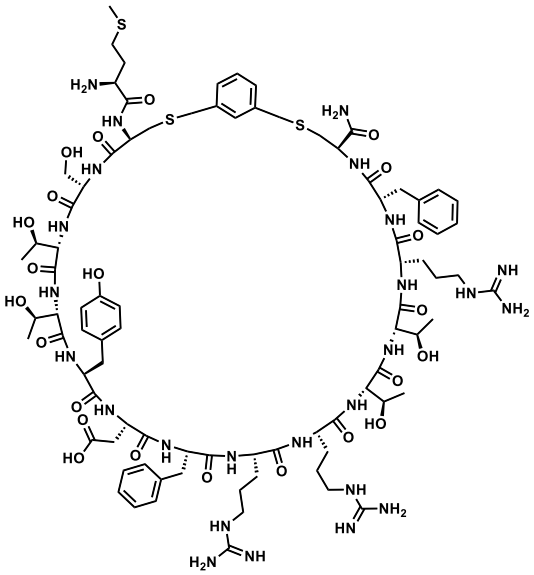


Figure S63. ESI-LC-MS characterization of cyclized peptide DBX-2.

Peptide: Cyclized DBX-2

Sequence: MCSTTYDFRRTTRFC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{87}H_{129}N_{25}O_{23}S_3$, 1987.8855; found, 1987.8909.

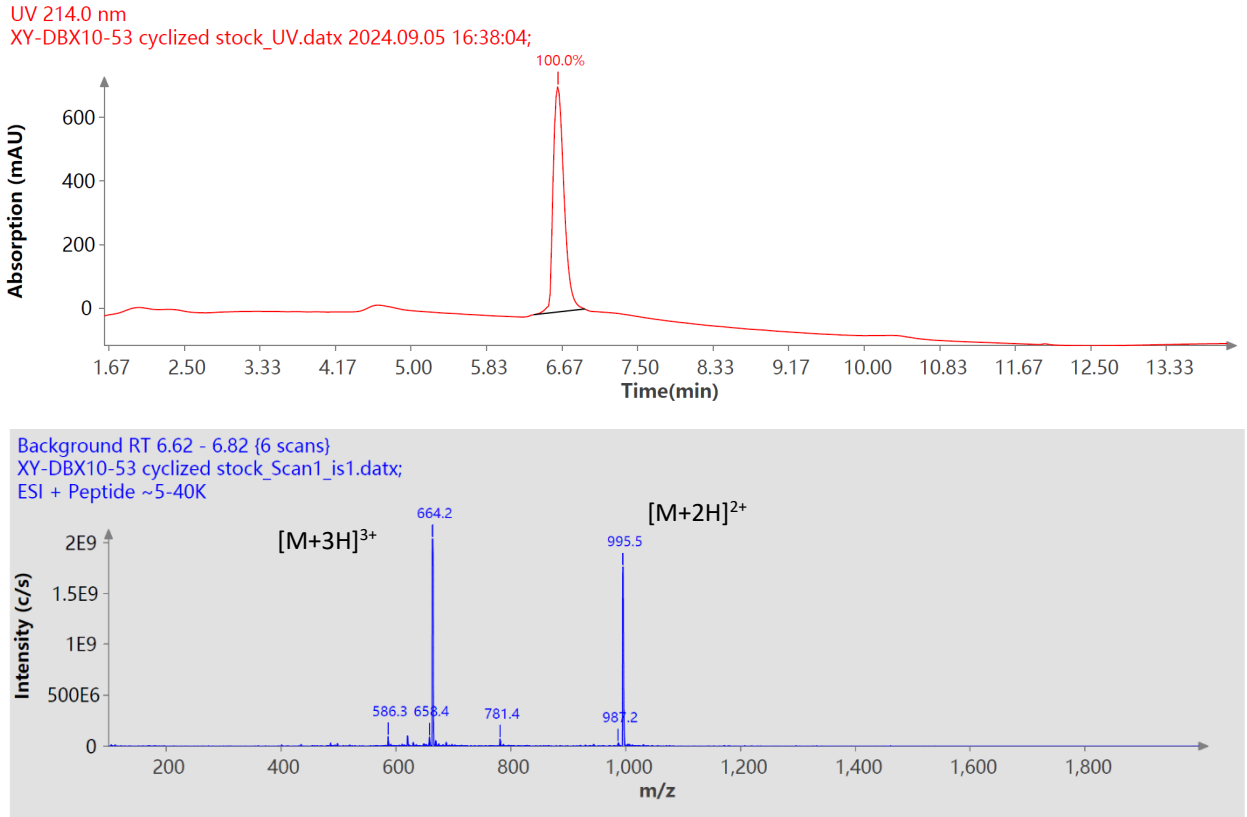
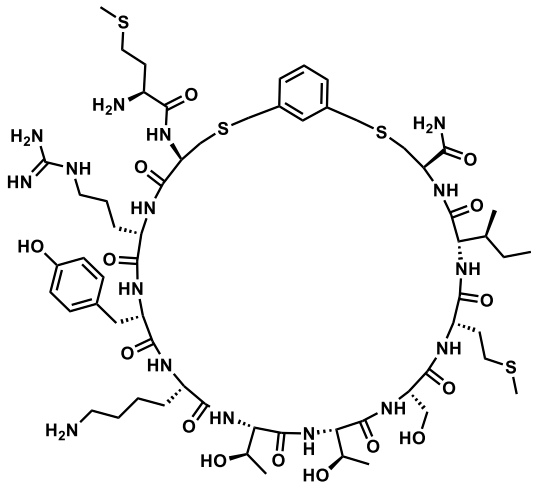


Figure S64. ESI-LC-MS characterization of cyclized peptide DBX-2.

Peptide: Cyclized DBX-3

Sequence: MCRYKTTSMIC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{62}H_{99}N_{15}O_{16}S_4$, 1437.6277; found, 1437.6516.

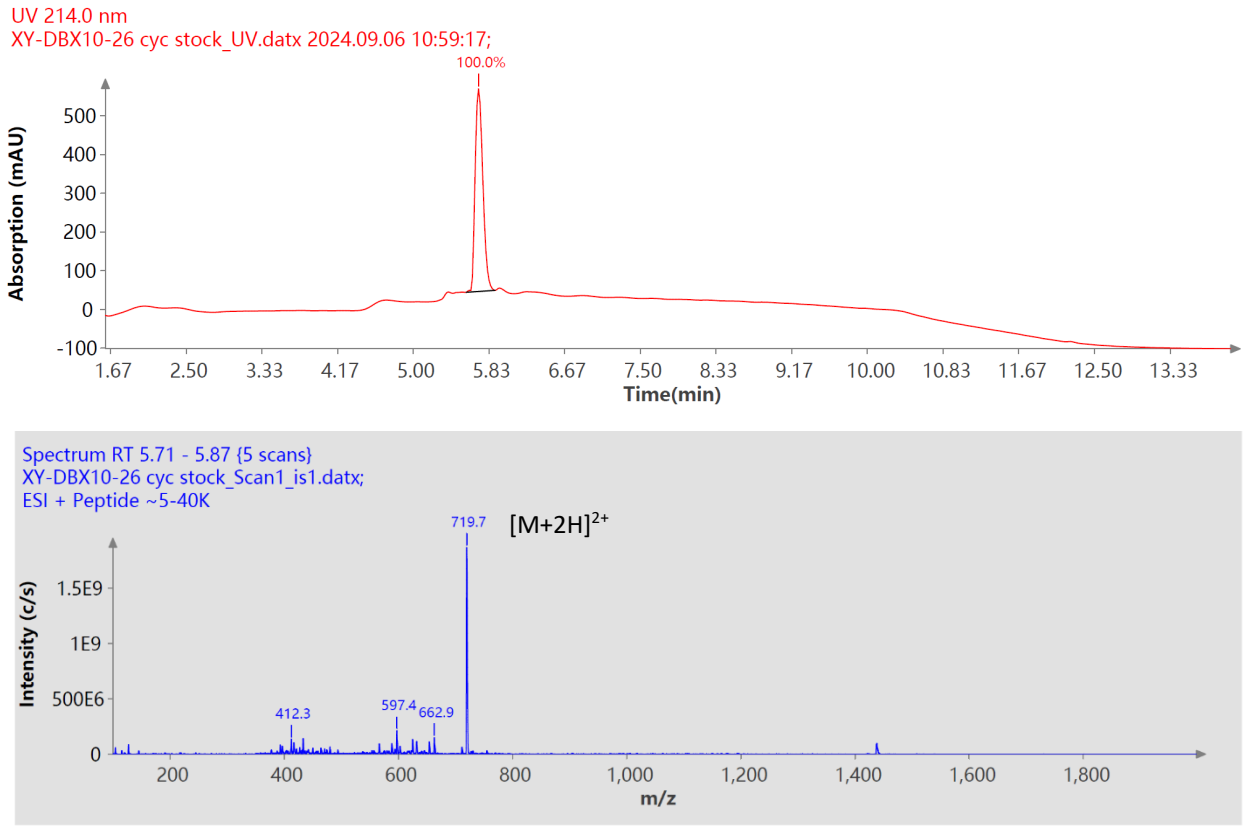
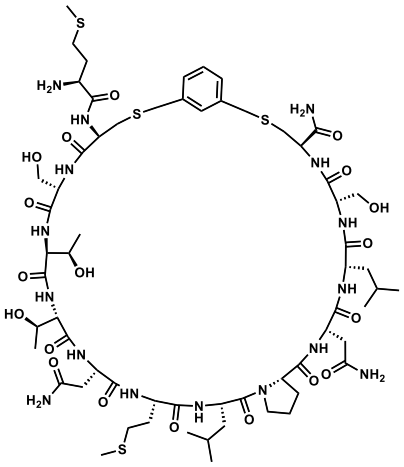


Figure S65. ESI-LC-MS characterization of cyclized peptide DBX-3.

Peptide: Cyclized DBX-4

Sequence: MCSTTNMLPNLSC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{63}H_{102}N_{16}O_{19}S_4$, 1514.6390; found, 1514.6396.

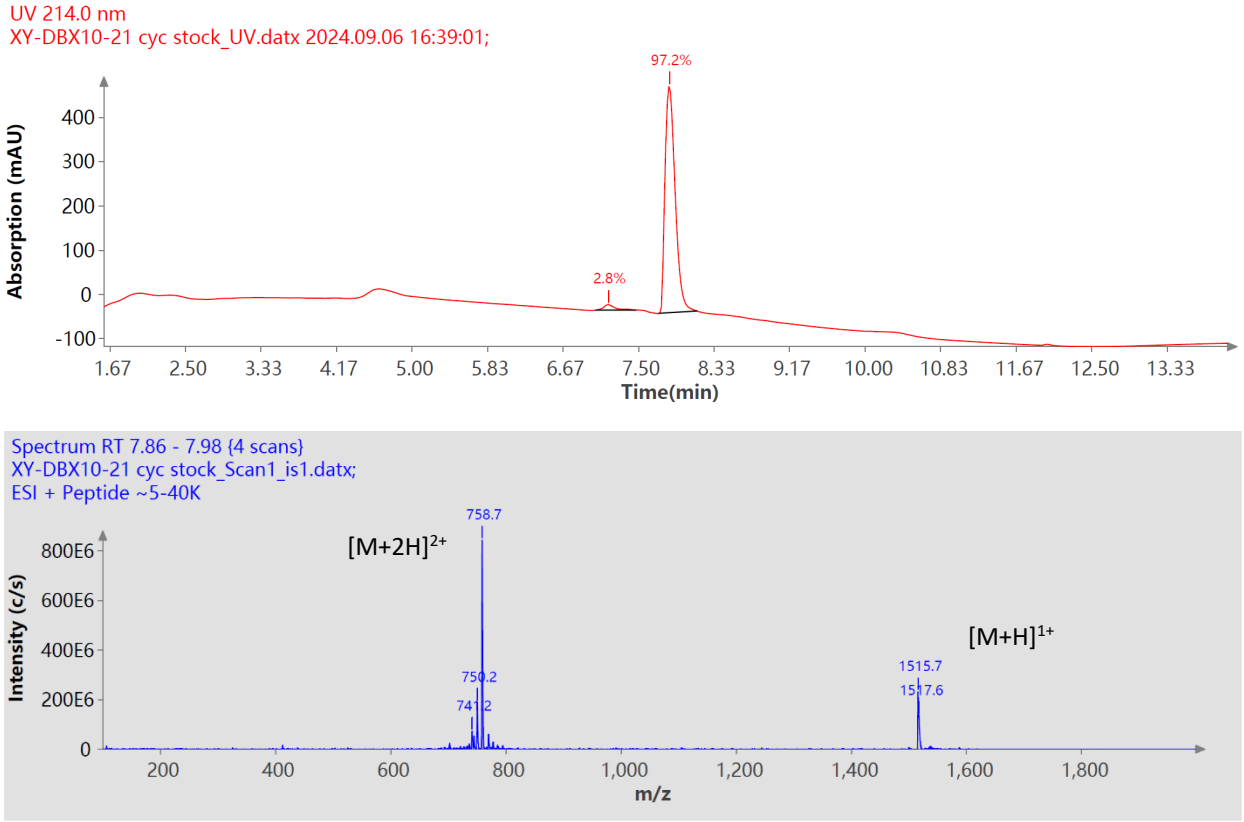
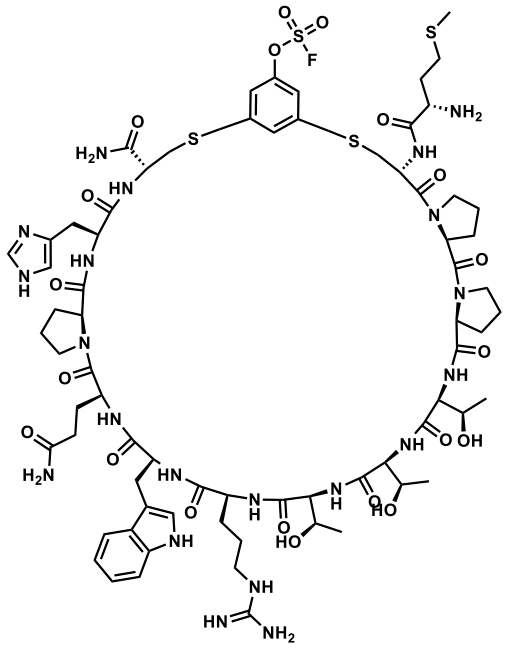


Figure S66. ESI-LC-MS characterization of cyclized peptide DBX-4.

Peptide: FS-1

Sequence: MCPPTTTRWQPHC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{74}H_{106}FN_{21}O_{20}S_4$, 1755.6790; found, 1755.6742.

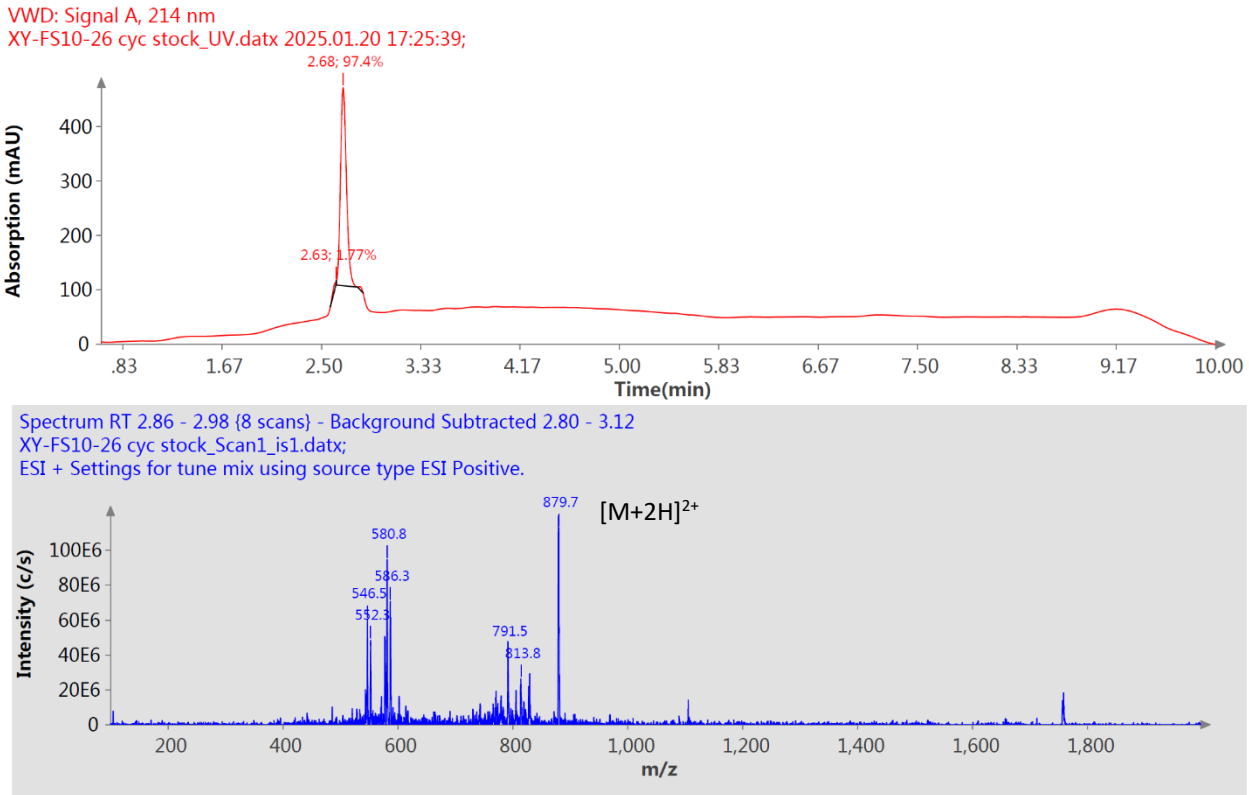
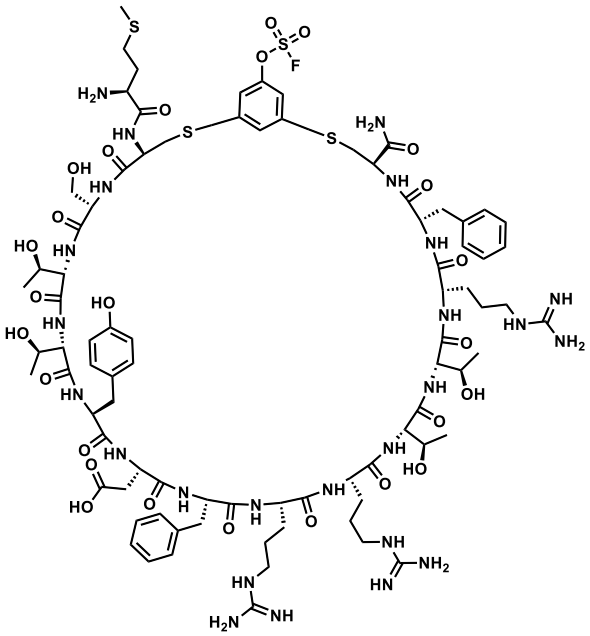


Figure S67. ESI-LC-MS characterization of cyclized peptide FS-1.

Peptide: Cyclized FS-2

Sequence: MCSTTYDFRRTTRFC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{87}H_{128}FN_{25}O_{26}S_4$, 2085.8329; found, 2085.8163.

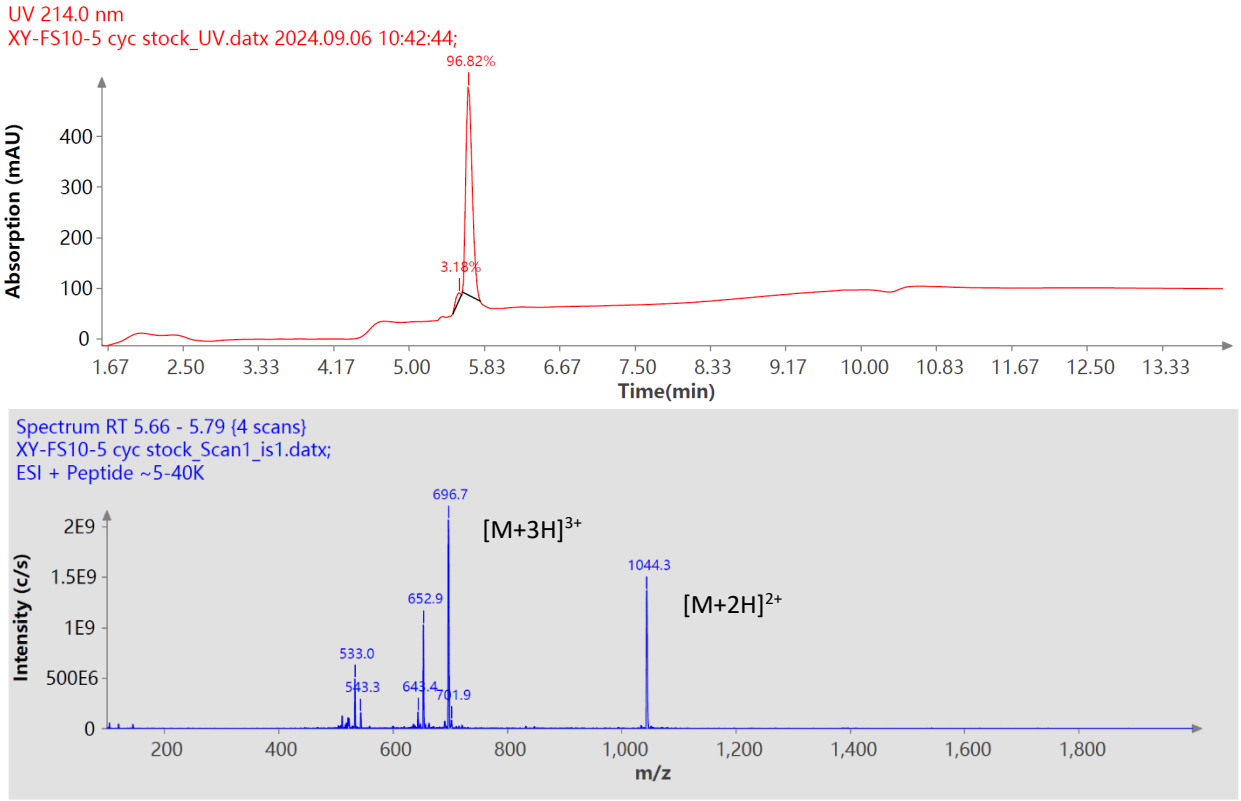
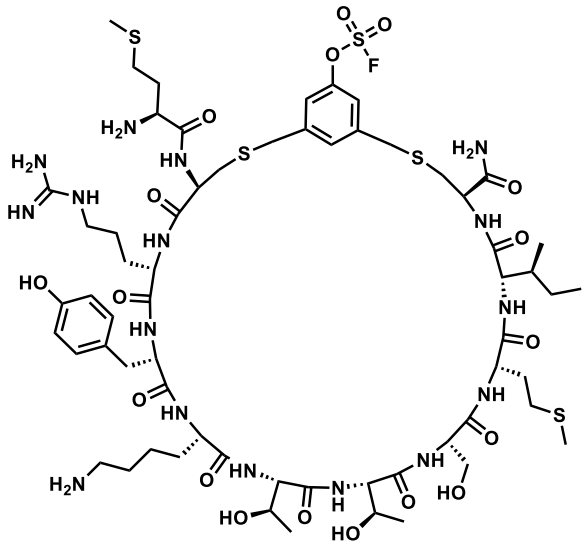


Figure S68. ESI-LC-MS characterization of cyclized peptide FS-2.

Peptide: FS-3

Sequence: MCRYKTTSMIC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{62}H_{99}FN_{16}O_{18}S_5$, 1534.5911; found, 1534.5932.

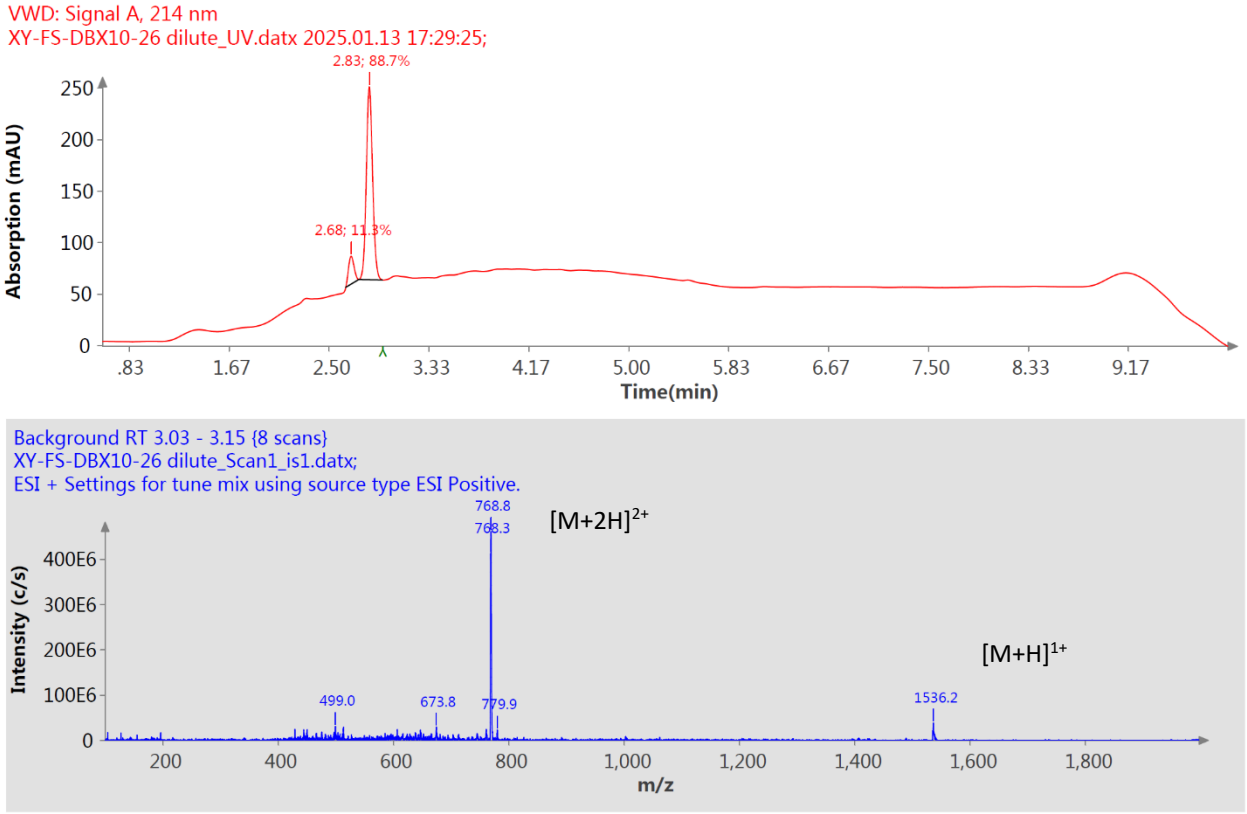
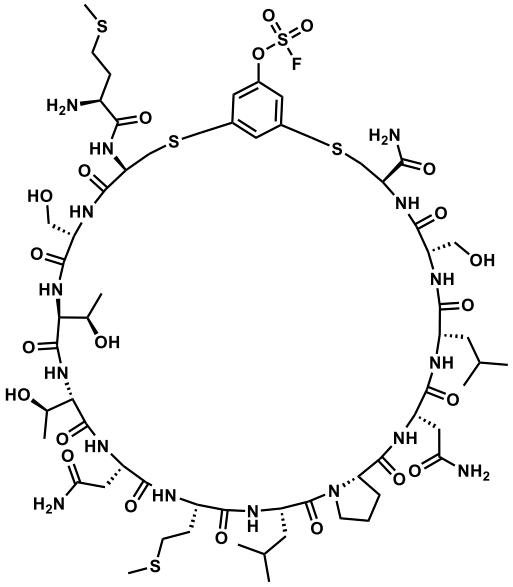


Figure S69. ESI-LC-MS characterization of cyclized peptide FS-3.

Peptide: FS-4

Sequence: MCSTTNMLPNLSC



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{63}H_{101}FN_{16}O_{22}S_5$, 1612.5864; found, 1612.5819.

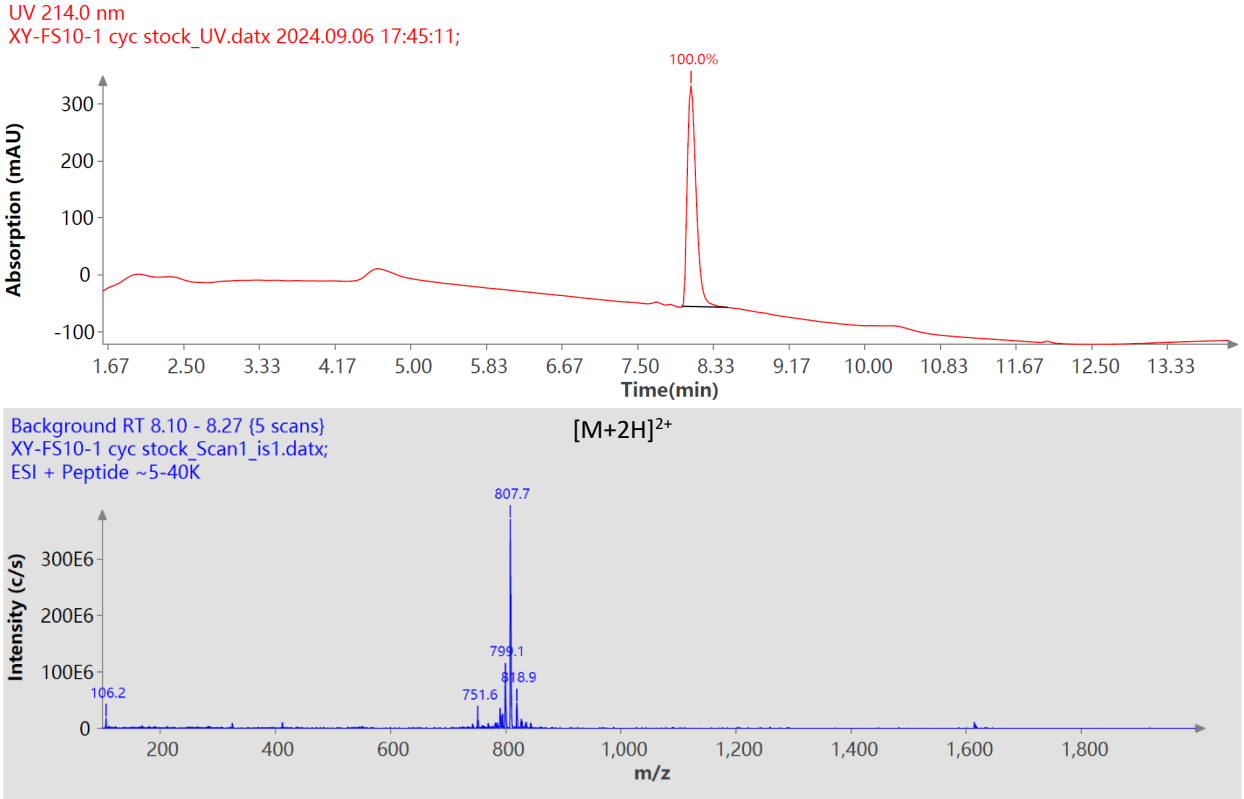
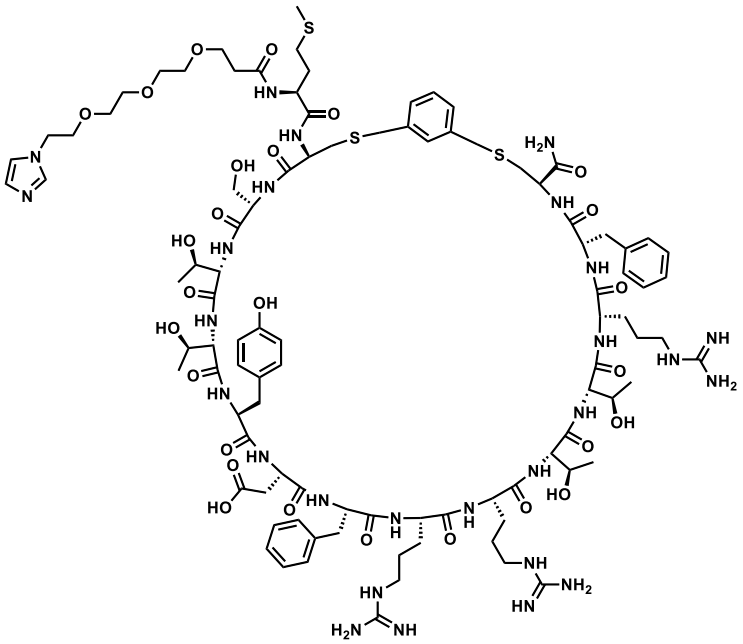


Figure S70. ESI-LC-MS characterization of cyclized peptide FS-4.

Peptide: Cyclized DBX-2-Im



m/z calculated for $C_{99}H_{147}N_{27}O_{27}S_3$, 2242.0122.

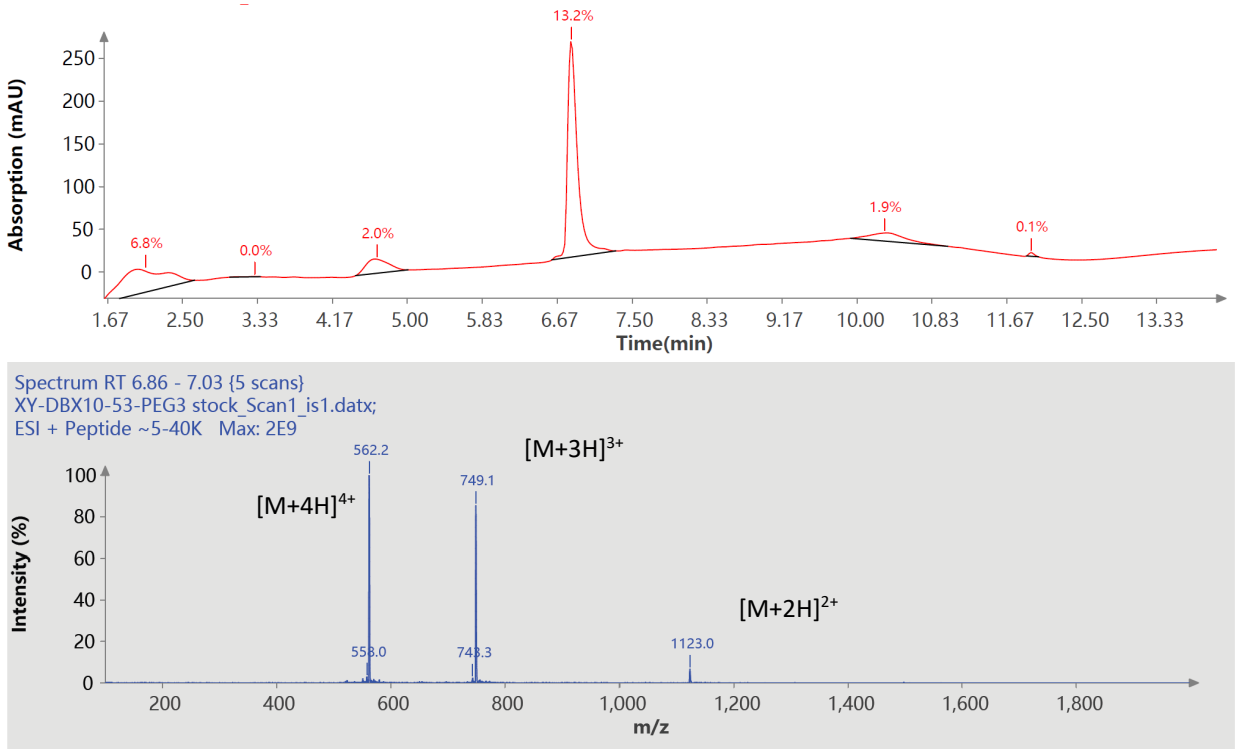
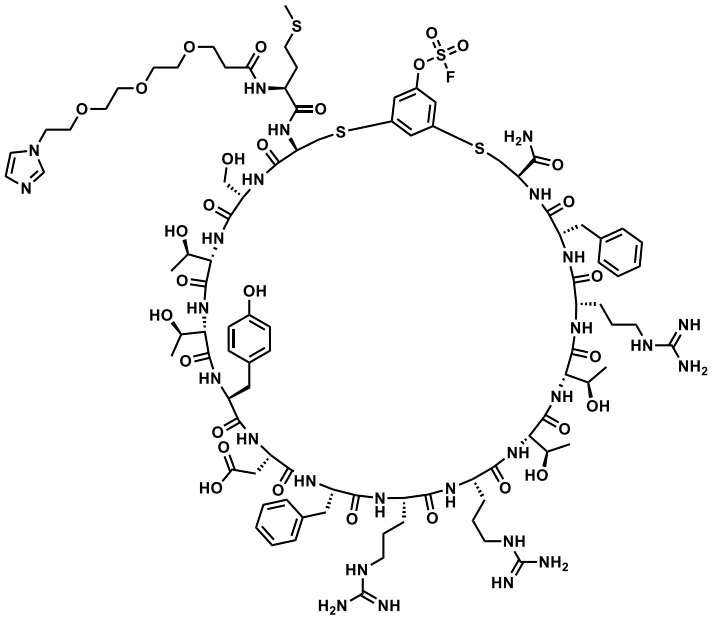


Figure S71. ESI-LC-MS characterization of cyclized peptide DBX-2-Im.

Peptide: Cyclized FS-2-Im



m/z calculated for $C_{99}H_{146}FN_{27}O_{30}S_4$, 2339.9596.

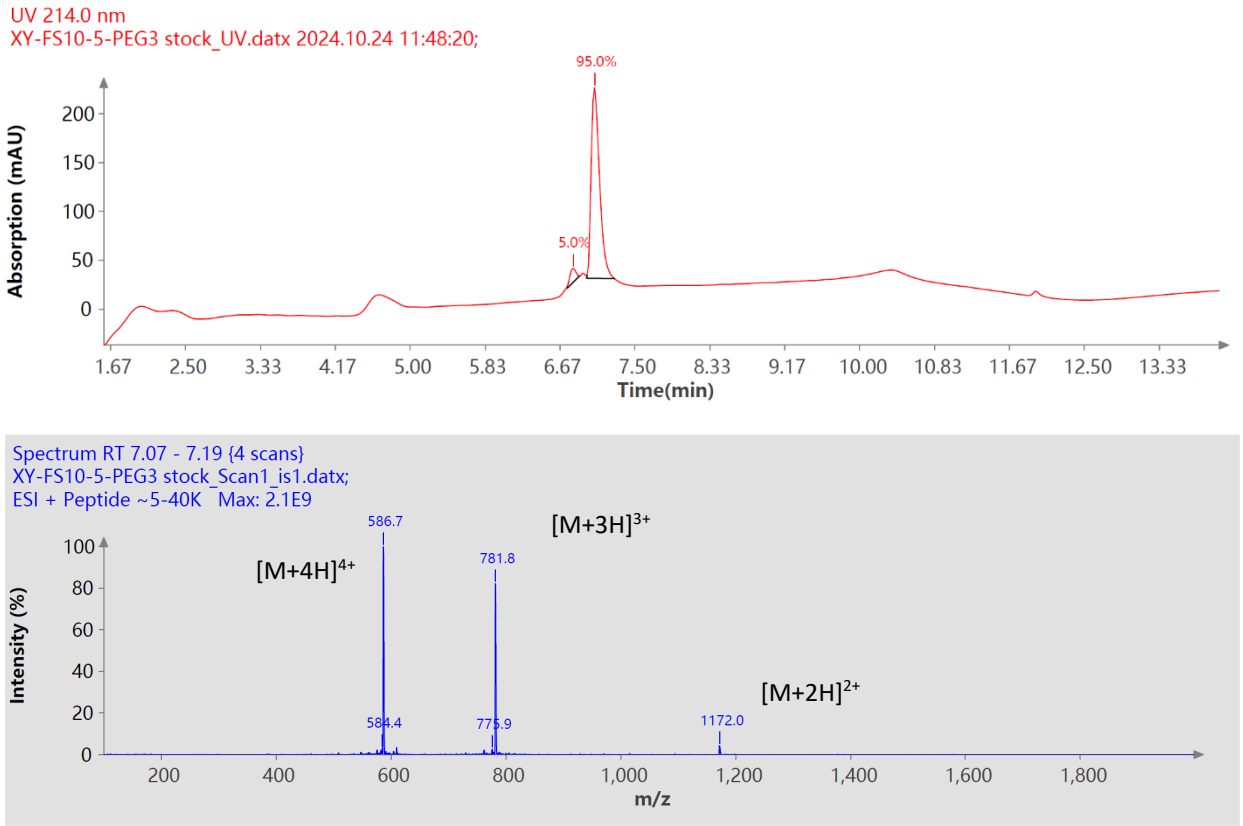
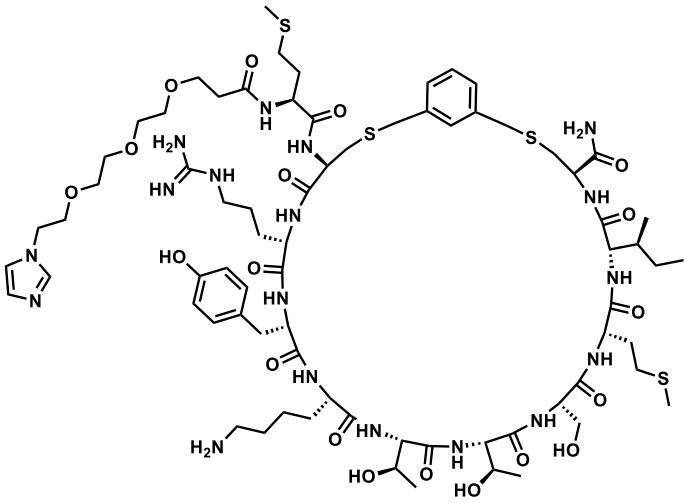


Figure S72. ESI-LC-MS characterization of cyclized peptide FS-2-Im.

Peptide: Cyclized DBX-3-Im



HR-MS (ESI-TOF, positive-ion mode): m/z calculated for $C_{74}H_{118}N_{18}O_{19}S_4$, 1690.7703; found, 1690.7692.

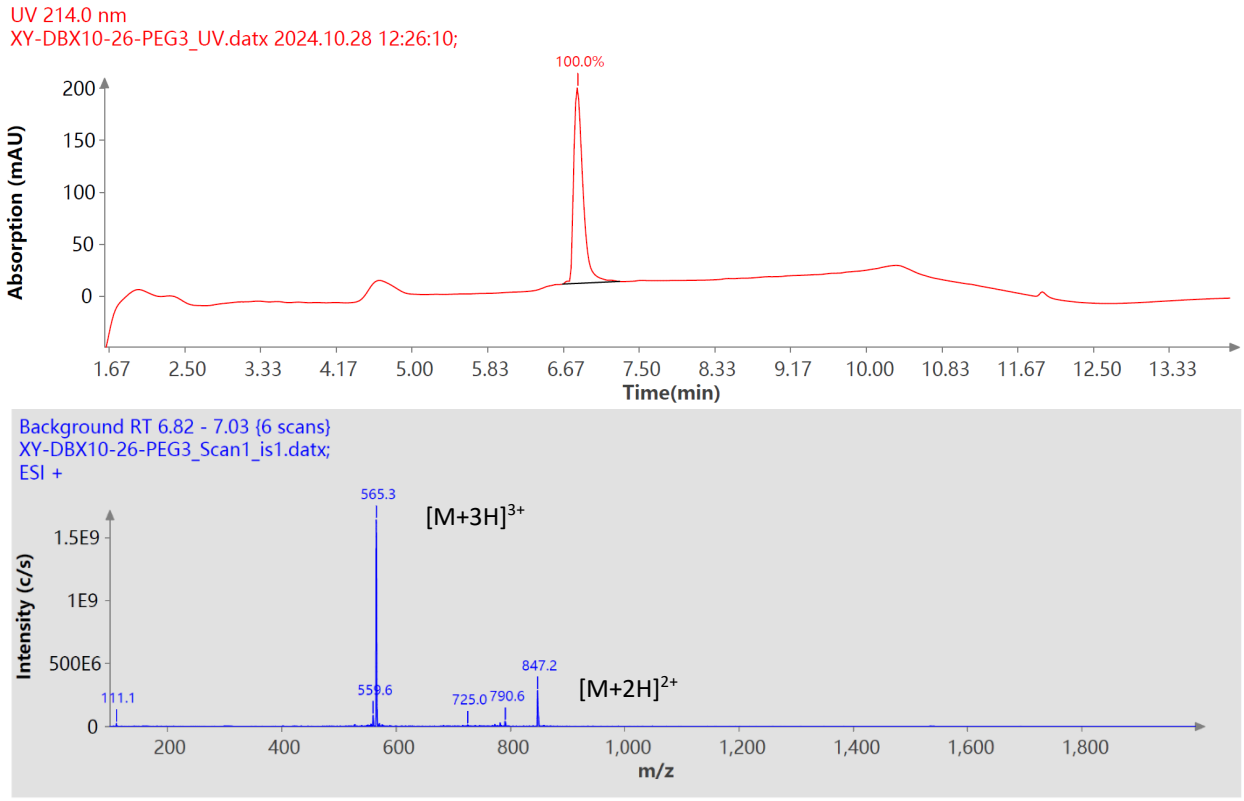
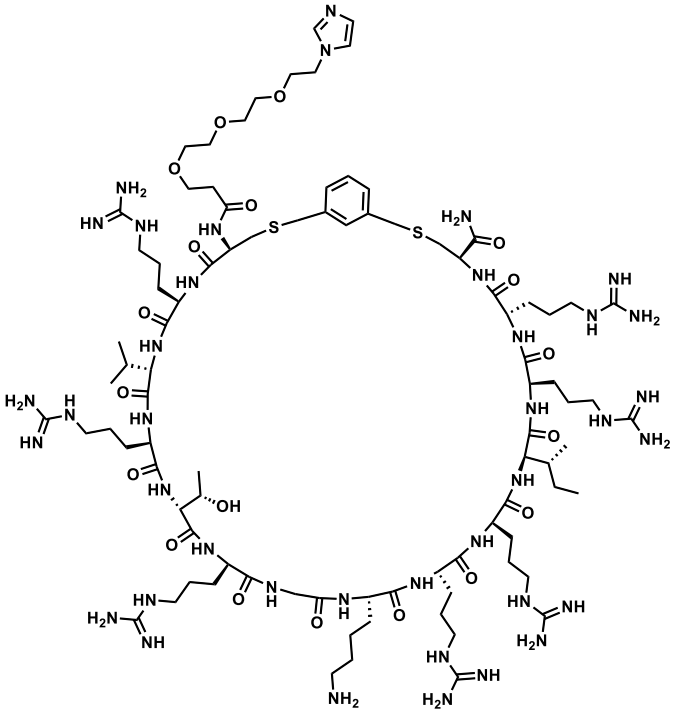


Figure S73. ESI-LC-MS characterization of cyclized peptide DBX-3-Im.

Peptide: Cyclized Tar-2-Im



m/z calculated for $C_{91}H_{163}N_{39}O_{19}S_2$, 2170.2429.

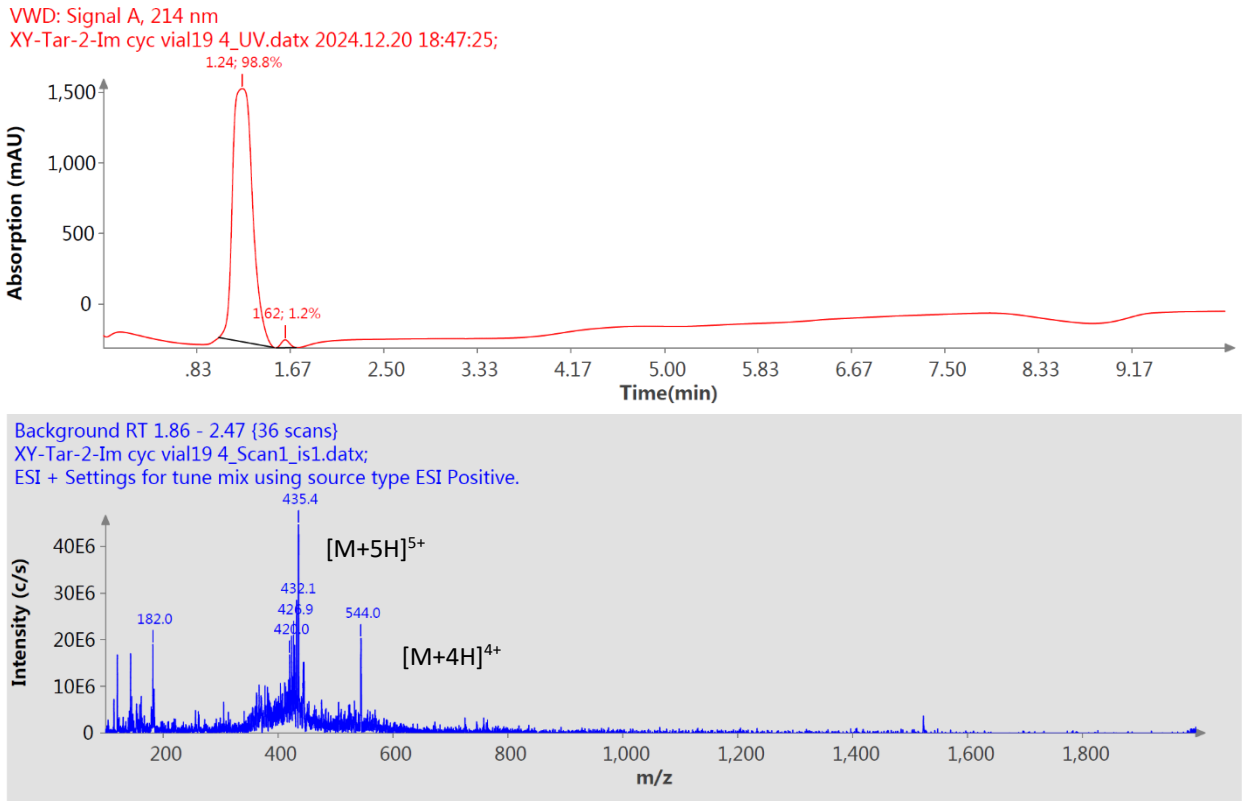


Figure S74. ESI-LC-MS characterization of cyclized Tar-2-Im.

REFERENCES

(1) Lan, T.; Peng, C.; Yao, X.; Chan, R. S. T.; Wei, T.; Rupanya, A.; Radakovic, A.; Wang, S.; Chen, S.; Lovell, S.; Snyder, S. A.; Bogyo, M.; Dickinson, B. C. Discovery of Thioether-Cyclized Macrocyclic Covalent Inhibitors by MRNA Display. *J Am Chem Soc* **2024**, *146* (34), 24053–24060. https://doi.org/10.1021/jacs.4c07851.

(2) Rebollo, I. R.; Sabisz, M.; Baeriswyl, V.; Heinis, C. Identification of Target-Binding Peptide Motifs by High-Throughput Sequencing of Phage-Selected Peptides. *Nucleic Acids Res* **2014**, *42* (22). https://doi.org/10.1093/nar/gku940.

(3) Rogers, D.; Hahn, M. Extended-Connectivity Fingerprints. *J Chem Inf Model* **2010**, *50* (5), 742–754. https://doi.org/10.1021/ci100050t.

(4) Van Der Maaten, L.; Hinton, G. Visualizing Data Using T-SNE. *Journal of machine learning research* **2008**, 2579-2605.

(5) Wilkinson, K. A.; Merino, E. J.; Weeks, K. M. Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE): Quantitative RNA Structure Analysis at Single Nucleotide Resolution. *Nat Protoc* **2006**, *1* (3), 1610–1616. https://doi.org/10.1038/nprot.2006.249.

(6) Fang, L.; Velema, W. A.; Lee, Y.; Xiao, L.; Mohsen, M. G.; Kietrys, A. M.; Kool, E. T. Pervasive Transcriptome Interactions of Protein-Targeted Drugs. *Nat Chem* **2023**, *15* (10), 1374–1383. https://doi.org/10.1038/s41557-023-01309-8.

(7) Gibaut, Q. M. R.; Bush, J. A.; Tong, Y.; Baisden, J. T.; Taghavi, A.; Olafson, H.; Yao, X.; Childs-Disney, J. L.; Wang, E. T.; Disney, M. D. Transcriptome-Wide Studies of RNA-Targeted Small Molecules Provide a Simple and Selective r(CUG)Exp Degrader in Myotonic Dystrophy. *ACS Cent Sci* **2023**, *9* (7), 1342–1353. https://doi.org/10.1021/acscentsci.2c01223.

(8) Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A. J.; Bambrick, J.; Bodenstein, S. W.; Evans, D. A.; Hung, C. C.; O'Neill, M.; Reiman, D.; Tunyasuvunakool, K.; Wu, Z.; Žemgulytė, A.; Arvaniti, E.; Beattie, C.; Bertolli, O.; Bridgland, A.; Cherepanov, A.; Congreve, M.; Cowen-Rivers, A. I.; Cowie, A.; Figurnov, M.; Fuchs, F. B.; Gladman, H.; Jain, R.; Khan, Y. A.; Low, C. M. R.; Perlin, K.; Potapenko, A.; Savy, P.; Singh, S.; Stecula, A.; Thillaisundaram, A.; Tong, C.; Yakneen, S.; Zhong, E. D.; Zielinski, M.; Žídek, A.; Bapst, V.; Kohli, P.; Jaderberg, M.; Hassabis, D.; Jumper, J. M. Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3. *Nature* **2024**, *630* (8016), 493–500. https://doi.org/10.1038/s41586-024-07487-w.

(9) Wohlwend, J.; Corso, G.; Passaro, S.; Reveiz, M.; Leidal, K.; Swiderski, W.; Portnoi, T.; Chinn, I.; Silterra, J.; Jaakkola, T.; Barzilay, R. Boltz-1: Democratizing Biomolecular Interaction Modeling. *BioRxiv* **2024**. https://doi.org/10.1101/2024.11.19.624167.

(10) Garofalo, A.; Parat, A.; Bordeianu, C.; Ghobril, C.; Kueny-Stotz, M.; Walter, A.; Jouhannaud, J.; Begin-Colin, S.; Felder-Flesch, D. Efficient Synthesis of Small-Sized Phosphonated Dendrons: Potential Organic Coatings of Iron Oxide Nanoparticles. *New Journal of Chemistry* **2014**, *38* (11), 5226–5239. https://doi.org/10.1039/c4nj00654b.