A D-Amino Acid at the N-terminus of a Protein Abrogates its Degradation by the N-End Rule Pathway

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Supplementary Information

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Supplemental Data:

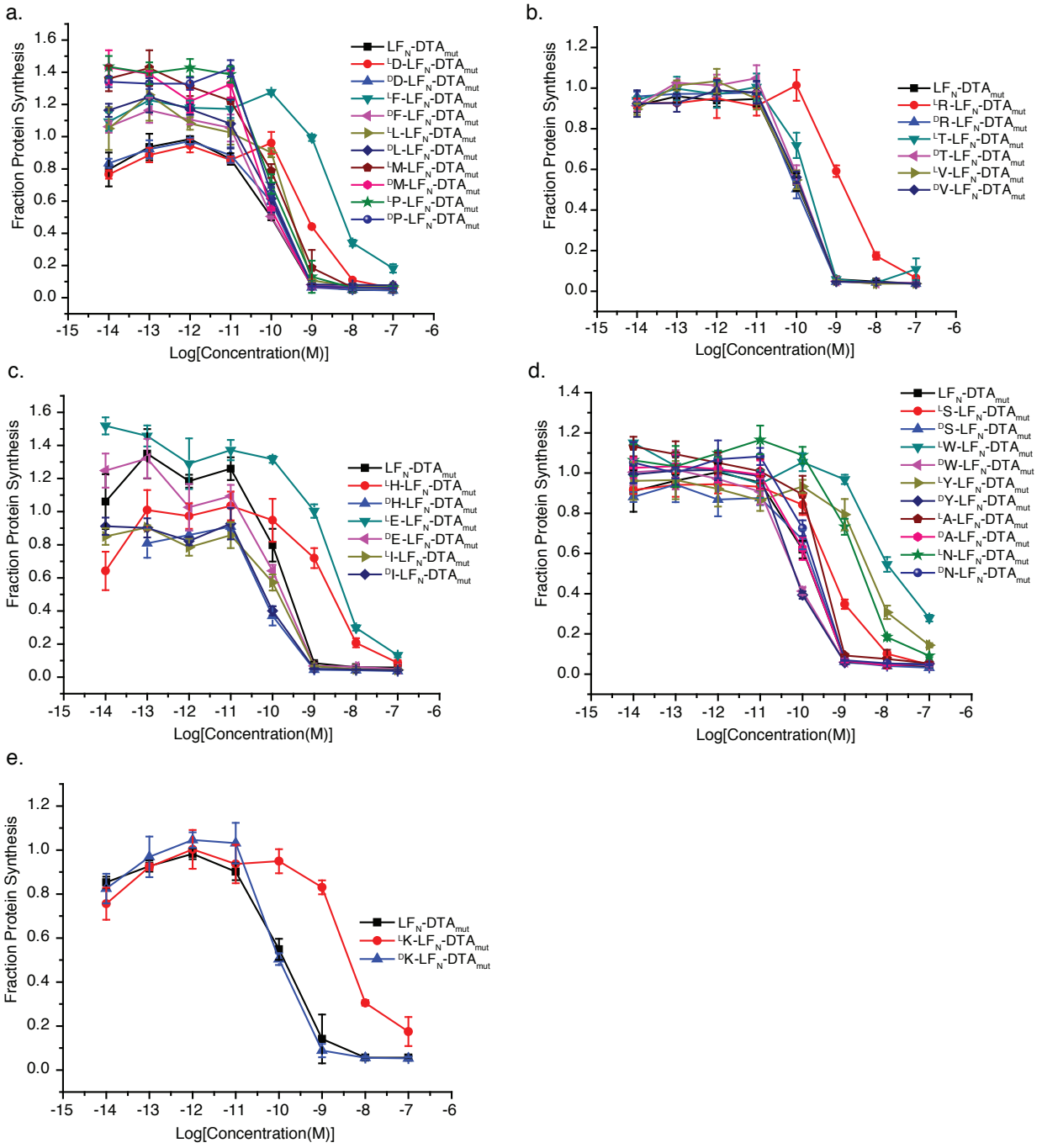


Figure S1 Translocation of X-LF_N-DTA_{mut} constructs were analyzed by the protein synthesis inhibition assay in CHO-K1 cells treated for 6 hours in the presence of 20 nM PA.

X-LF_N-DTA_{mut}, X represents:	EC_{50} (nM)
^{D}Y	0.074
^{D}W	0.077
^{D}M	0.078
^{D}F	0.084
^{D}H	0.085
^{D}I	0.089
^{D}P	0.092
^{L}P	0.094
^{D}K	0.094
^{D}L	0.099
^{D}R	0.10
^{D}E	0.11
^{D}T	0.11
^{D}V	0.11
^{L}V	0.11
^{D}A	0.12
^{L}M	0.13
^{L}I	0.13
^{D}D	0.13
LF_N-DTA_{mut}	0.13
^{D}N	0.15
^{L}T	0.16
^{D}S	0.17
^{L}A	0.21
^{L}L	0.21
^{L}S	0.56
^{L}D	0.96
^{L}N	1.6
^{L}R	1.6
^{L}E	1.9
^{L}H	2.4
^{L}F	2.5
^{L}K	3.7
^{L}Y	3.8
^{L}W	5.5

Table S1 EC_{50} values for X-LF_N-DTA_{mut} constructs translocated in CHO-K1 cells from Supplementary Figure S1.

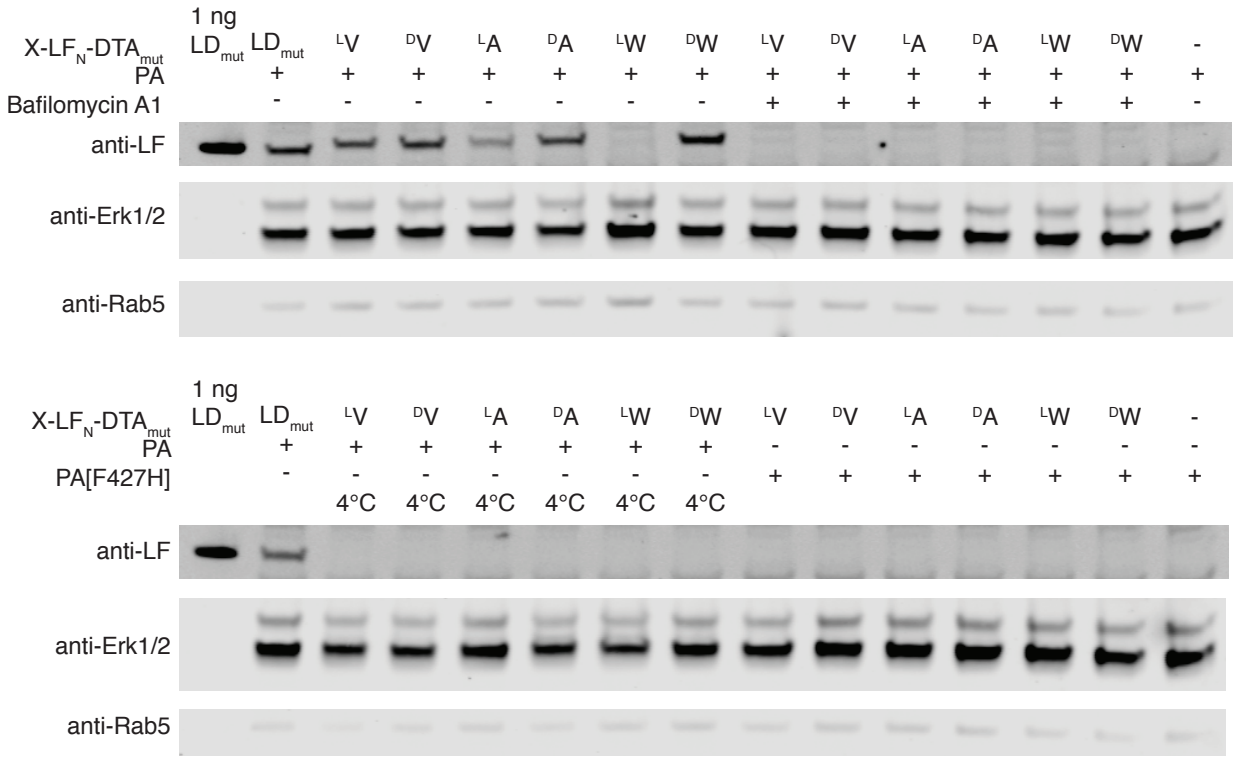


Figure S2 Western blot analysis of X-LF$_N$-DTA$_{mut}$ constructs in CHO-K1 cells in the presence of 200 nM Bafilomycin A1, 20 nM PA[F427H], or incubated at 4°C over 6 hours.

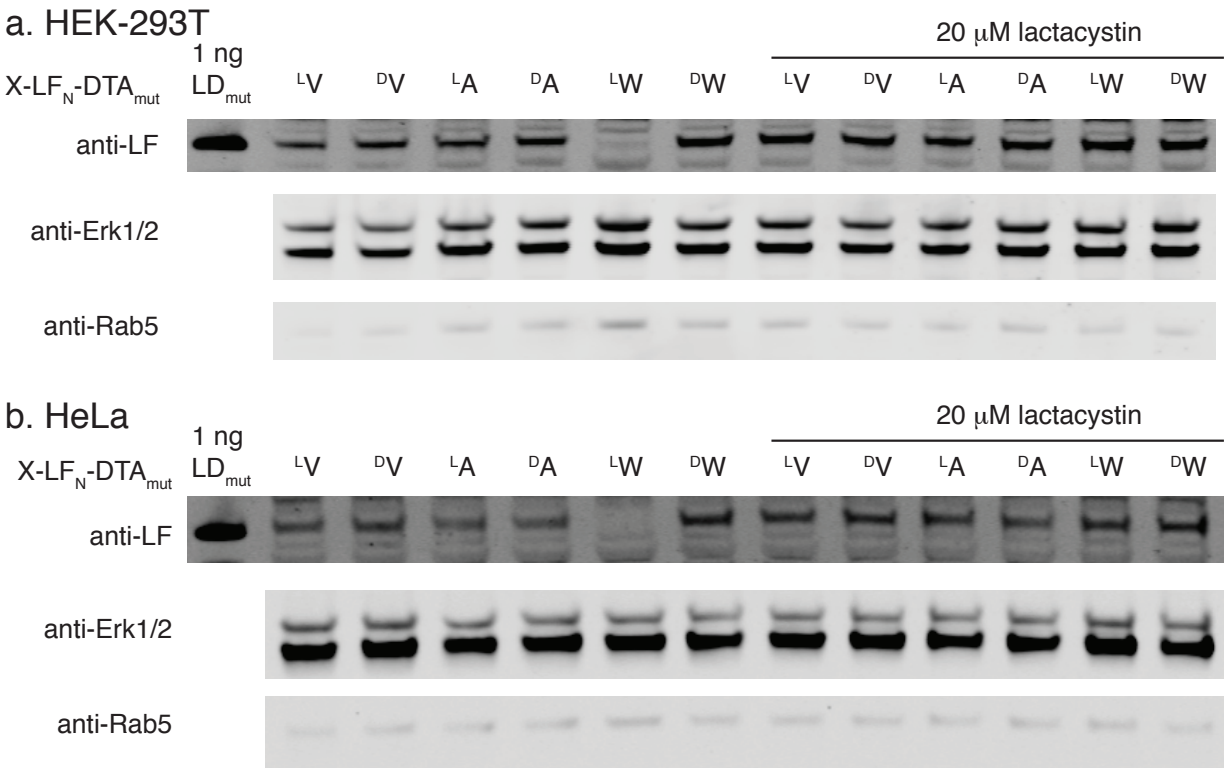


Figure S3 Western blot analysis of X-LF$_N$-DTA$_{mut}$ constructs delivered into HEK-293T (a) or HeLa (b) cells in the presence of 20 nM PA over 6 hours.

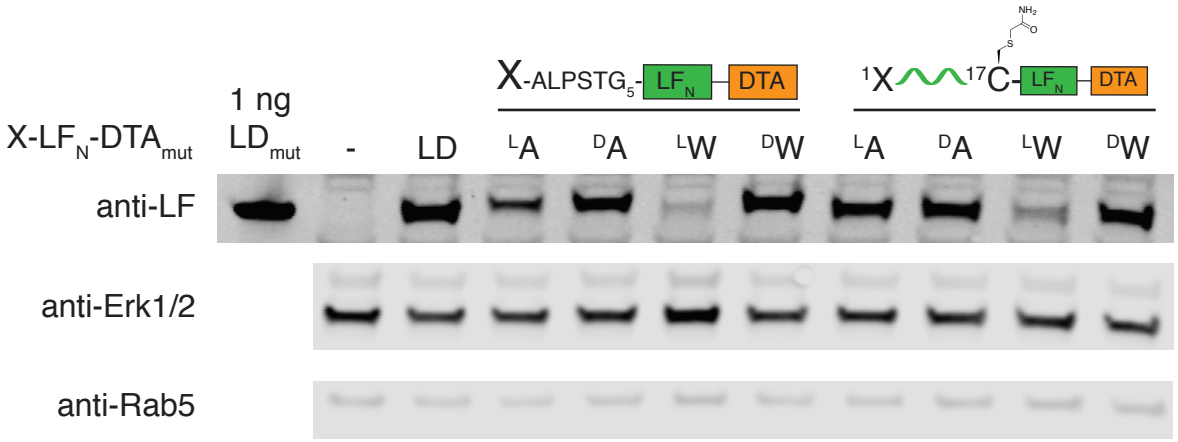


Figure S4 Sortagged (left) and native (right) X-LF_N-DTA_{mut} constructs (where X represents ^{L}A, ^{D}A, ^{L}W, and ^{D}W) were translocated in CHO-K1 cells in the presence of 20 nM PA for 6 hours then analyzed by western blot.

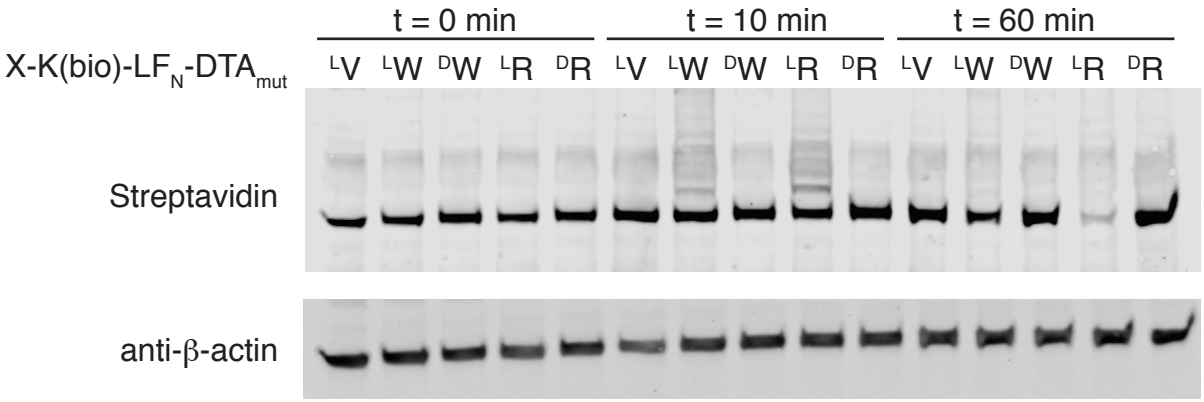


Figure S5 In vitro degradation of X-Kbio-LF$_N$-DTA$_{mut}$, where X represents ^{L}V, ^{L}W, ^{D}W, ^{L}R, and ^{D}R, in rabbit reticulocyte lysate over time.

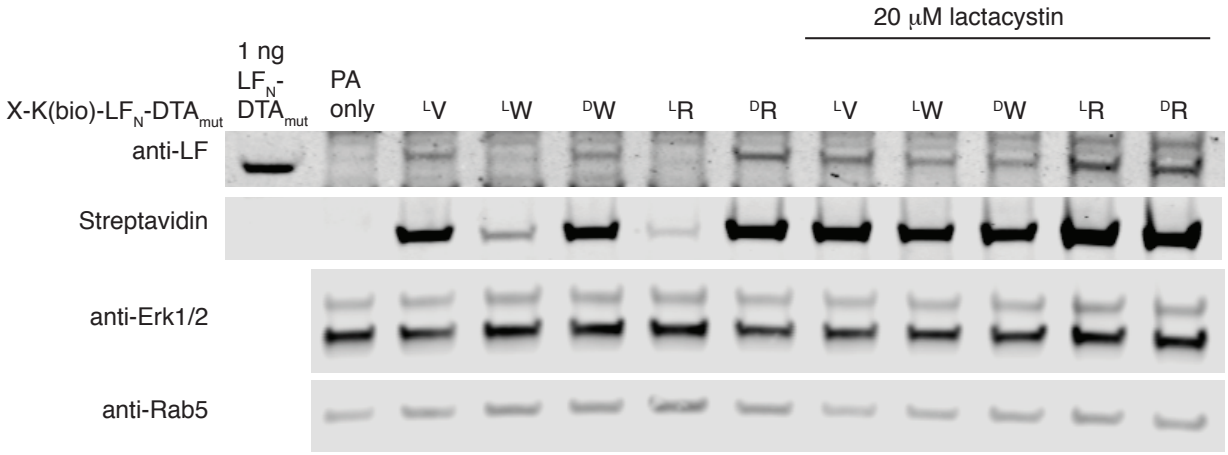


Figure S6 ^{L}V-, $^{L/D}$W-, and $^{L/D}$R-K(bio)-LF$_N$-DTA$_{mut}$ were translocated into CHO-K1 cells in the presence of 20 nM PA for 6 hours then analyzed by western blot. As a proteasomal inhibitor, 20 μM lactacystin was used.

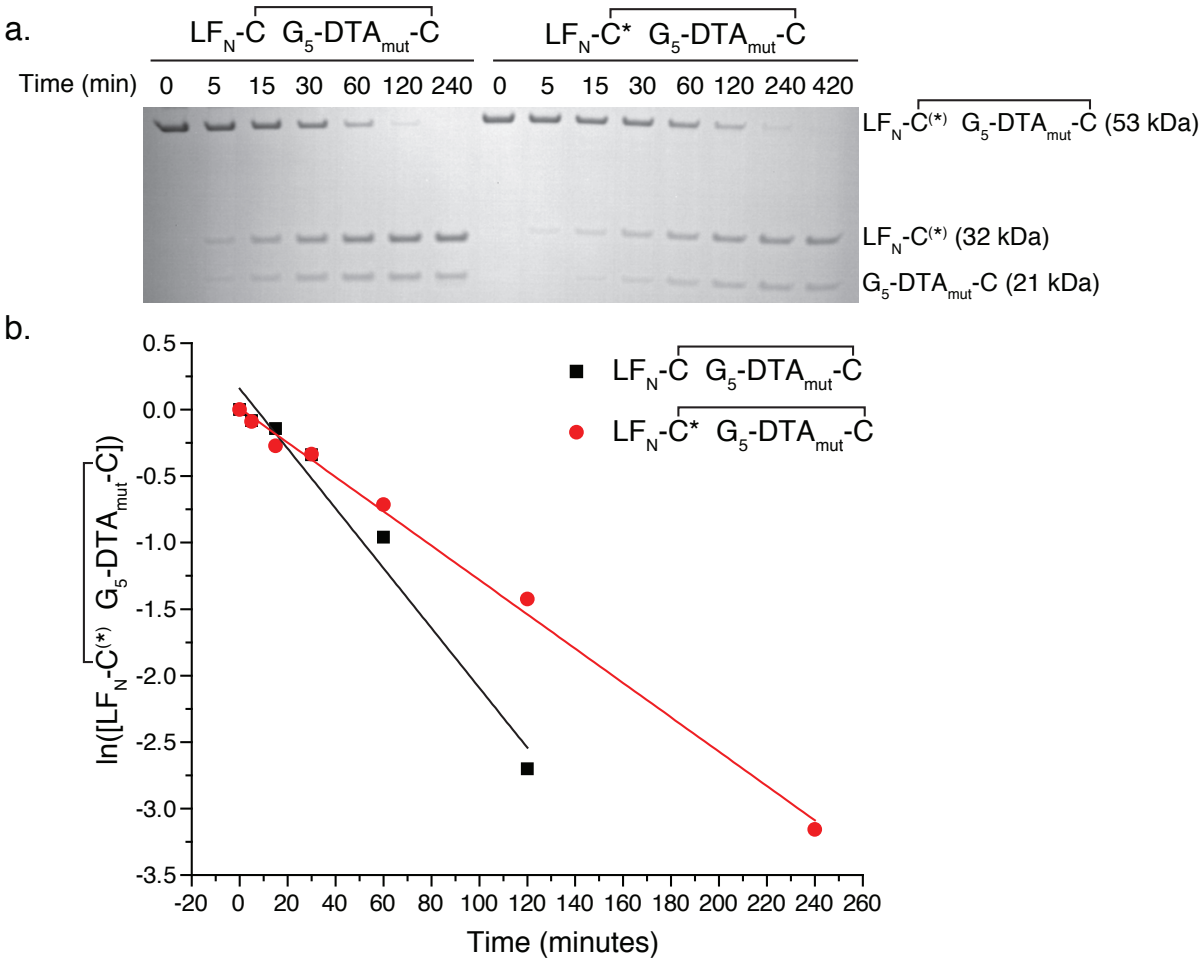


Figure S7 Rate of reduction for **1-X**. a. Coomassie-stained SDS Page gel of 1 μM **1-X** (disulfide) or **1-X** (hindered disulfide) incubated with 1 mM DTT in 20 mM Tris pH 7.5 and 150 mM NaCl incubated at 37 °C. b. The first order reaction rates were determined by plotting ln([**1-X**]) over time. The rate of reduction (k_{obs}) for **1-X** (disulfide) was 0.0225 min^{-1} (R^2 = 0.972) and **1-X** (hindered disulfide) was 0.0116 min^{-1} (R^2 = 0.995).

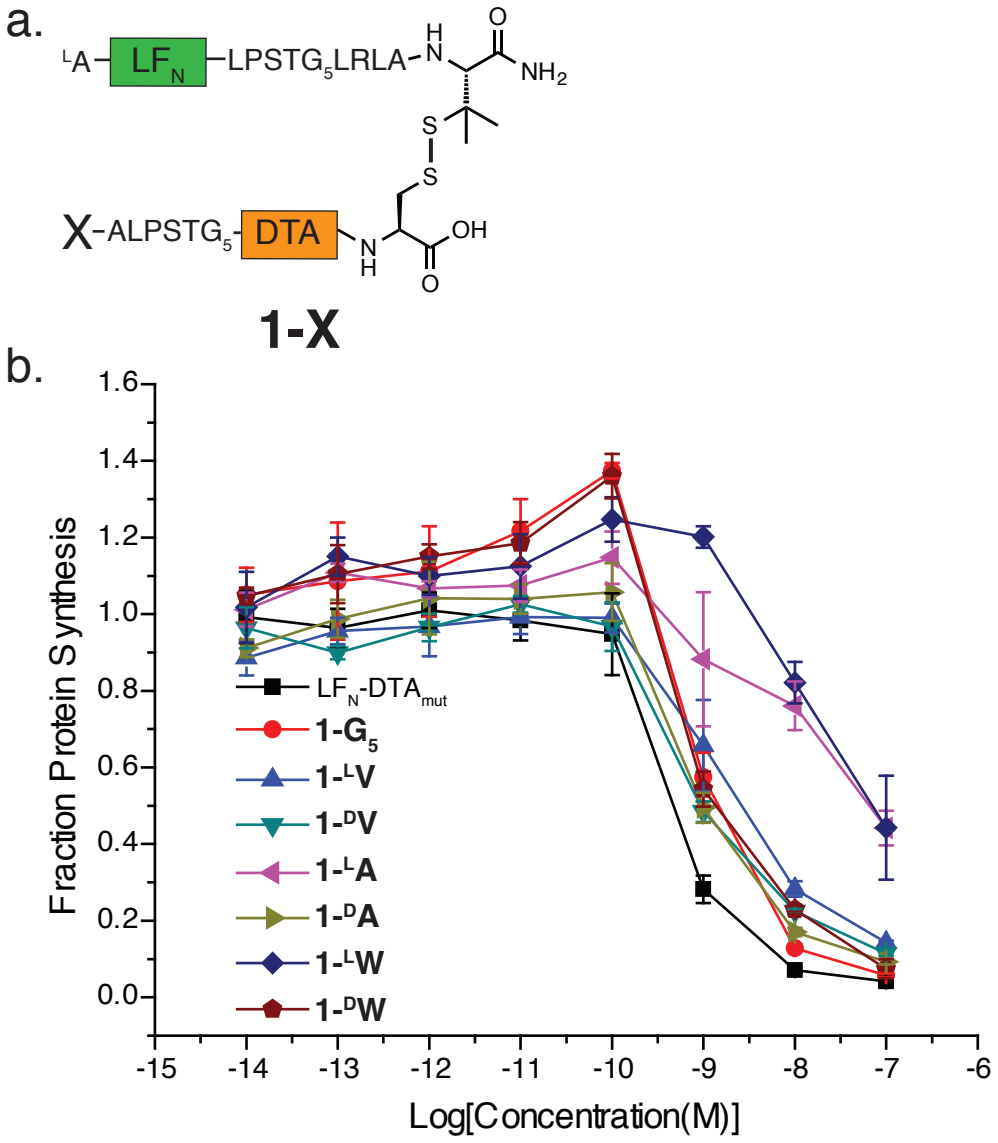


Figure S8 Delivery of **1-X**. a. Molecular composition of **1-X**. b. CHO-K1 cells were treated with ten-fold dilutions of **1-X** in the presence of 20 nM PA then analyzed by protein synthesis inhibition assay. The results indicated that X-DTA$_{mut}$ was stabilized by one N-terminal D-amino acid after cleavage from LF$_N$.

Supplemental Experimental Procedures:

Protein Expression and Purification

G_5-LF_N-DTA_{mut}, LF_N-LPSTGG-H_6, G_5-DTA_{mut}-C, ^{17}C-LF_N-DTA_{mut}, LF_N-DTA-LPSTGG-H_5, G_5-$Duf5_{C2}$, and G_5-$Duf5_{C2}$C were expressed in BL21(DE3) *E. coli* cells. The cells were grown at 37 °C to an OD_{600} ~0.6-1.0. The proteins were induced with 0.4 mM IPTG overnight at 30 °C. After induction, the cells were pelleted and resuspended in 20 mM Tris pH 7.5 and 150 mM NaCl with protease inhibitor cocktail (Roche), DNaseI (Roche), and lysozyme. The cells were sonicated three times for 20 seconds on ice then spun down for 30 minutes at 35,000 g and 4 °C. The lysate was purified over a HisTrap FF NiNTA column (GE Healthcare) pre-equilibrated with 20 mM Tris pH 8.5 and 150 mM NaCl. Each protein was loaded onto the column then washed with the equilibration buffer and then with 20 mM Tris pH 8.5, 500 mM NaCl, and 40 mM imidazole. Finally, each protein was eluted in buffer containing 20 mM Tris pH 8.5, 500 mM NaCl, and 500 mM imidazole. The protein elutions were desalted using a HiLoad 26/10 desalt column (GE Healthcare) into 20 mM Tris pH 7.5 and 150 mM NaCl. After desalting the proteins, the native N-termini were obtained by cleaving the SUMO protein fusion. For SUMO cleavage, 1 μg SUMO protease was added per mg protein at room temperature. The cleaved sample was run over a second NiNTA column in order to obtain the pure protein in the flow through and buffer wash.

Wild-type PA and PA[F427H] were expressed in the periplasm of E. coli BL21 (DE3) cells and purified by anion exchange chromatography followed by size exclusion chromatography.

Fmoc Solid Phase Peptide Synthesis

All peptides were synthesized using Fmoc fast flow solid phase peptide synthesis (SPPS). All peptides were synthesized on a 0.1 mmol scale on aminomethyl resin with the Rink amide linker. Side-chain protection for the amino acids included: Arg(Pbf), Asn(Trt), Glu(OtBu), Lys(Boc), Lys(Alloc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). For each coupling, 1 mmol (10 eq) amino acid was dissolved in 0.4 M HBTU or HATU in DMF and diisopropylethylamine was used as the activating reagent. Peptides were synthesized according to the protocol in Simon, et al.[1] using 3 minute cycles at 60 °C of 40 sec amino acid coupling then 1 min DMF wash then 20 sec 20% piperidine in DMF (*v/v*) deprotection and finally 1 min DMF wash. After synthesis, peptides were cleaved from the resin with 94% TFA containing 2.5% EDT, 2.5% H_2O, and 1% TIPS (*v/v*) for 7 min at 60 °C. After cleavage, TFA was dried under $N_{2(g)}$, triturated three times with cold diethyl ether, dissolved in 50:50 A:B, and then lyophilized.

For biotinylated peptides, the allyloxycarbonyl (Alloc) protecting group was removed using 4.85 mmol phenylsilane and 39.5 μmol tetrakis(triphenylphosphine)palladium(0) in DCM for 20 min at RT. The resin was washed with DCM then DMF. Biotin (1 mmol) was coupled using 0.2 M HBTU in DMF for 20 min at RT.

Native Chemical Ligation of Native X-LF_N-DTA_{mut}

The following peptide-hydrazide was synthesized in preparation for native chemical ligation of native LF_N-DTA_{mut}: XGGHGDVGMHVKEKEK, where X represents ^{L}A, ^{D}A, ^{L}W, or ^{D}W.[2, 3] Additionally, ^{17}C-LF_N-DTA_{mut} was expressed and purified from *E. coli* using the expression and purification protocol described above. For native chemical ligation, each peptide

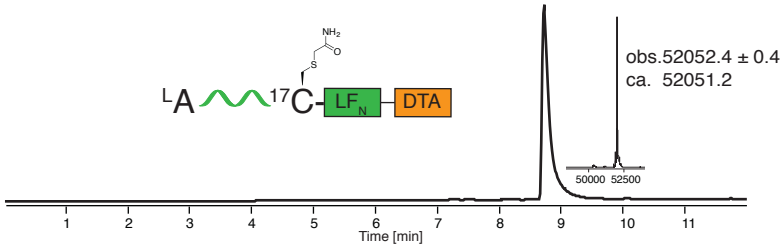
hydrazide (^{L}A, ^{D}A, ^{L}W, and ^{D}W-NHNH$_2$) was converted to a mercaptophenylacetic acid (MPAA) thioester using the following protocol.[4] Each peptide hydrazide (5 mM; 1 mg) was dissolved in 0.2 M phosphate buffer pH 3.0 then 1:10 dilution 0.2 M sodium nitrite (in 0.2 M phosphate buffer pH 3.0) was added to a final concentration of 20 mM sodium nitrite. The reaction was incubated for 10 minutes at -10 °C while stirring to form the peptide azide. After 10 minutes, the reaction was warmed to room temperature and the pH was adjusted to pH 7.0. A 1:1 dilution of 0.1 M MPAA (in 0.2 M phosphate buffer pH 7.0) was added to the reaction for a final concentration of 50 mM MPAA for 20 minutes at room temperature. Formation of the MPAA thioester peptides was confirmed by LCMS.

In one pot, 1 eq ^{17}C-LF$_N$-DTA$_{mut}$ (1 mg, 79.5 μM) was added to 28 eq of MPAA thioester (1 mg, 2.0 mM) and the pH was adjusted to pH 7.0 and incubated for 10 hours at room temperature to allow the NCL to go to completion. The reactions were monitored by LCMS. Upon completion of the reactions, 50 mM DTT was added to the samples for 20 minutes at room temperature to reduce any disulfides. The samples were buffer exchanged twice into PBS pH 7.0 to remove excess starting materials. The ligated proteins (^{L}A, ^{D}A, ^{L}W, and ^{D}W-LF$_N$-DTA$_{mut}$) were alkylated at the cysteine residue to form pseudoglutamine at position 17, which is asparagine in native LF. The cysteine residue was alkylated using 20 mM 2-bromoacetamide (in 0.2 M phosphate buffer pH 7.0) and pH was adjusted to pH 7.0 then incubated for 15 min then quenched with 50 mM sodium 2-mercaptoethane sulfonate (MESNa in 0.2 M phosphate buffer pH 7.0). The alkylated ligated proteins were desalted into 20 mM Tris pH 7.5, 150 mM NaCl using a HiTrap desalt column (GE Healthcare) then concentrated and analyzed by LCMS.

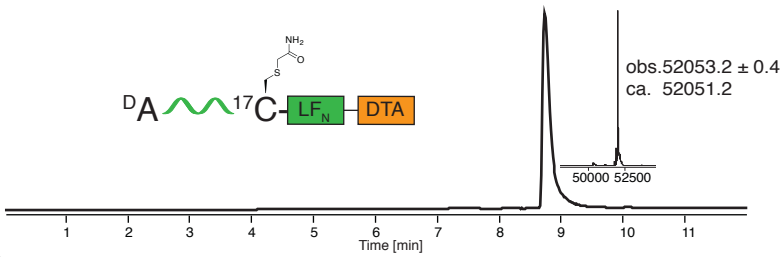
Synthesis of Hindered Disulfide Cleavable Linker

According to the ligation scheme in Figure S7, the peptide G$_3$LRLAC* was synthesized and purified then sortagged onto LF$_N$-LPSTGG under standard sortagging conditions to yield LF$_N$-C*. Protein cargos like DTA$_{mut}$ and DARPin were expressed with a C-terminal cysteine (G$_5$-cargo-C) for oxidation with C*. Each G$_5$-cargo-C protein was expressed and purified then activated with Ellman's reagent to form G$_5$-cargo-C-Ellman's. In order to obtain the desired N-terminus, XALPSTGG peptides, where X represents ^{L}V, ^{D}V, ^{L}A, ^{D}A, ^{L}W, and ^{D}W, were sortagged onto G$_5$-cargo-C-Ellman's using optimized conditions, giving a product yield between 90-95%. Finally, LF$_N$-C* and X-G$_5$-cargo-C-Ellman's were combined to form the hindered disulfide then purified to give the desired conjugates, **1-X** or **2-X**. The hindered disulfide comprised of cysteine and penicillamine was found to be 2-fold more stable to reduction than a cysteine-cysteine disulfide (Figure S8).

LCMS traces



^{L}A-LF$_{N}$-DTA NCL



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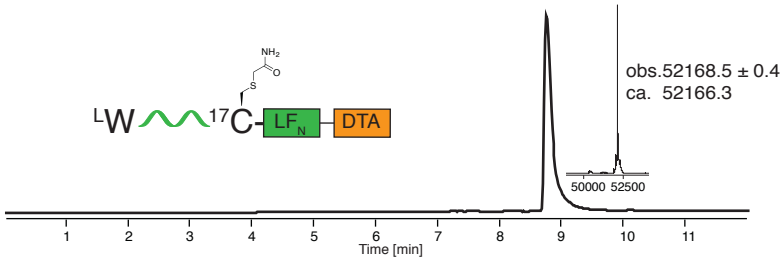
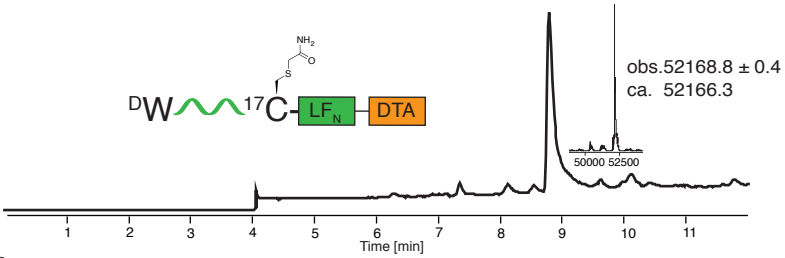
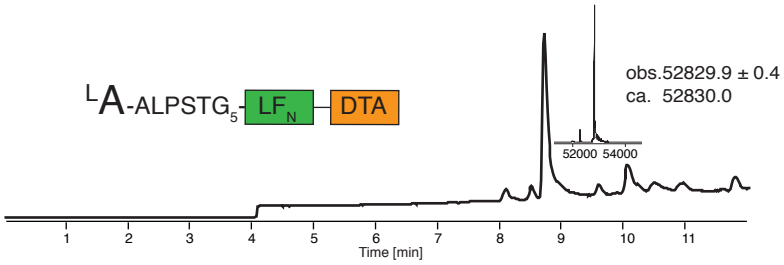


^{L}W-LF$_{N}$-DTA NCL



^{D}W-LF$_{N}$-DTA NCL



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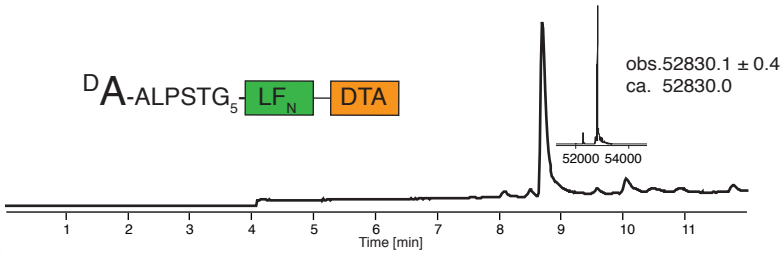

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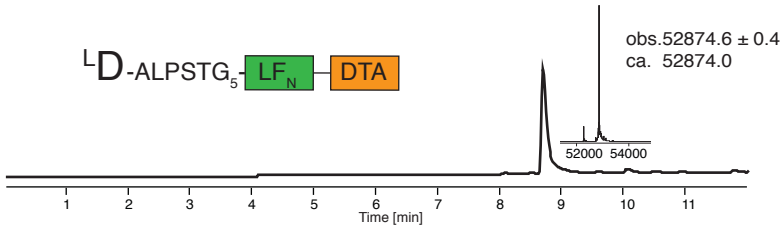

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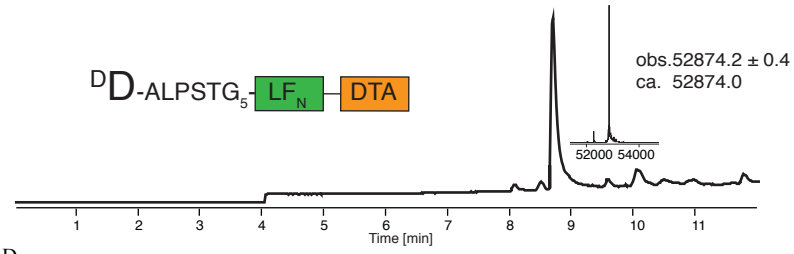

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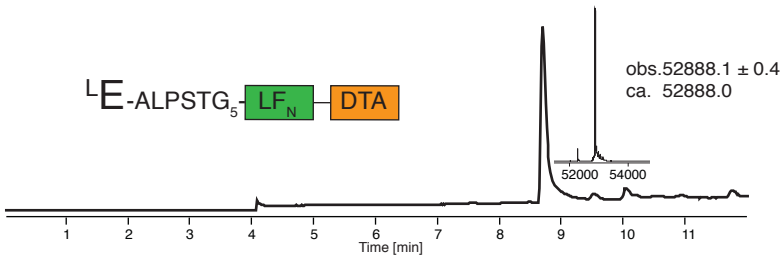

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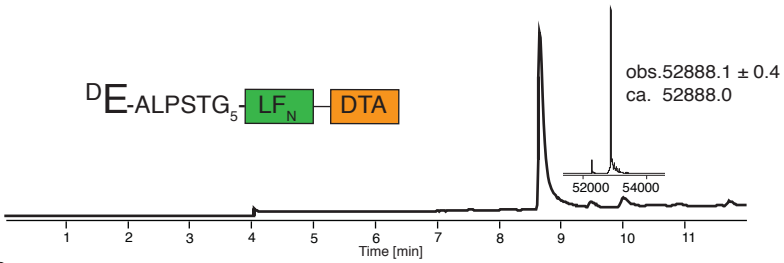

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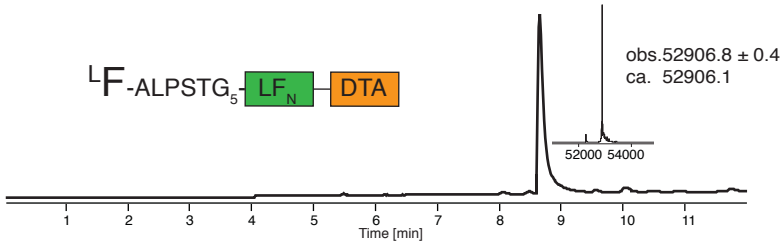

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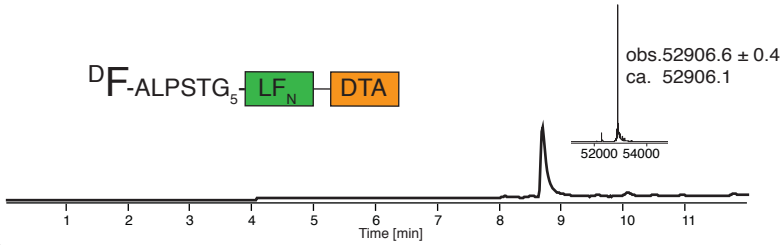

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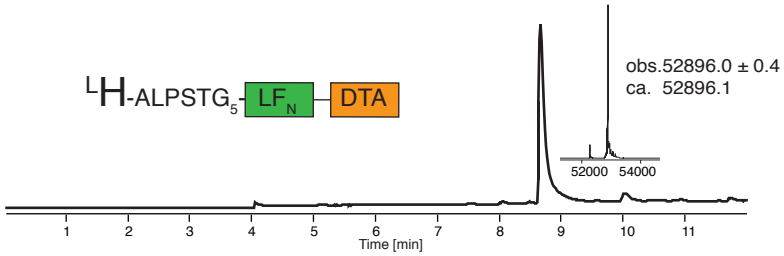

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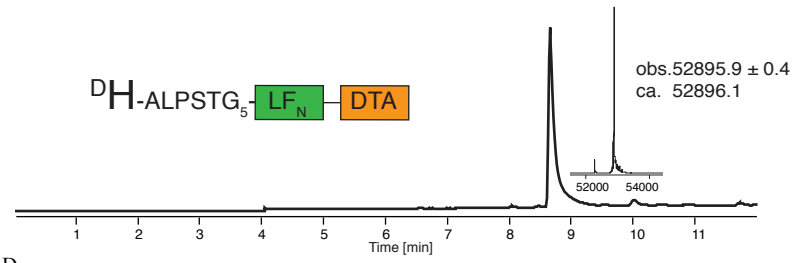

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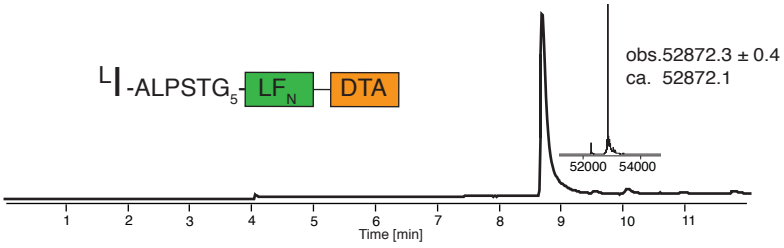

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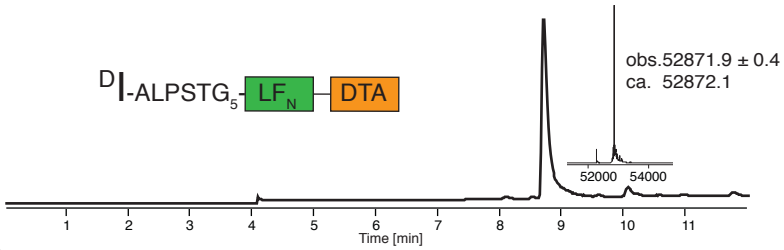

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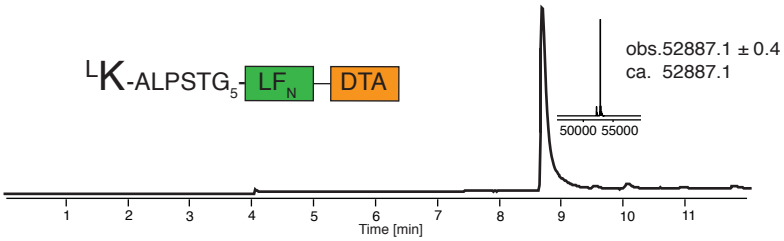

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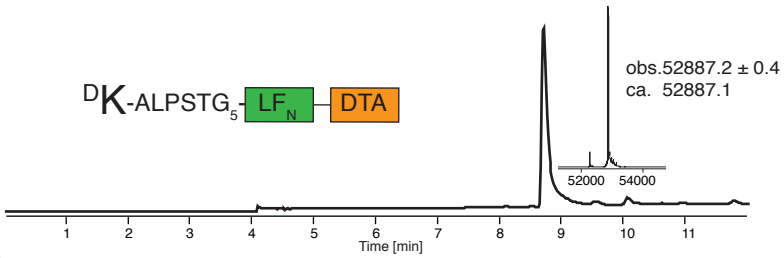

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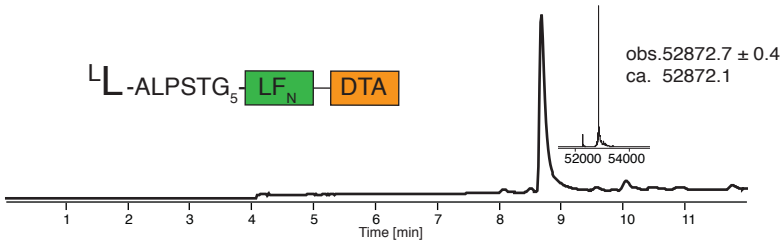

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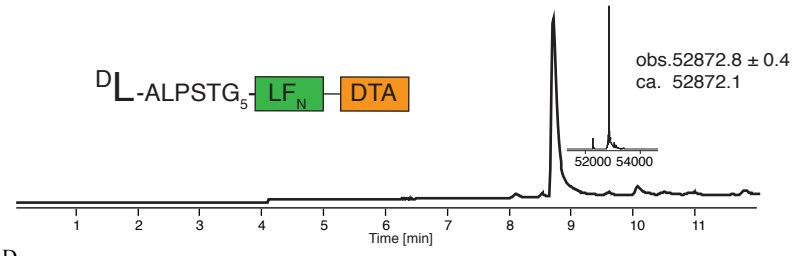

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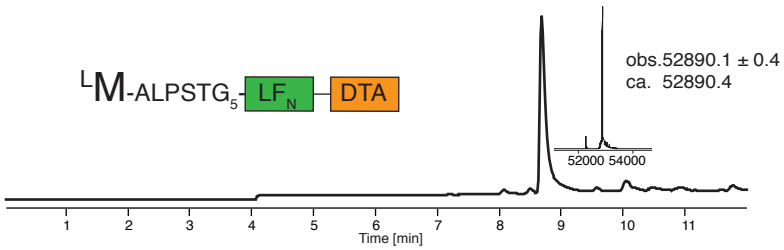

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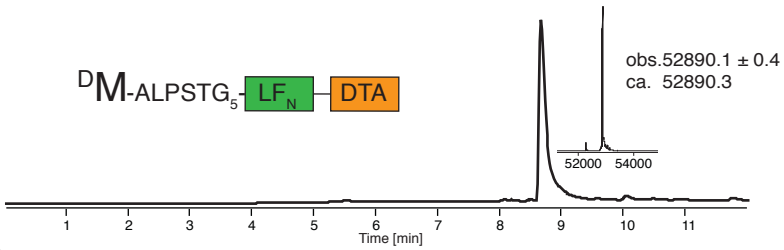

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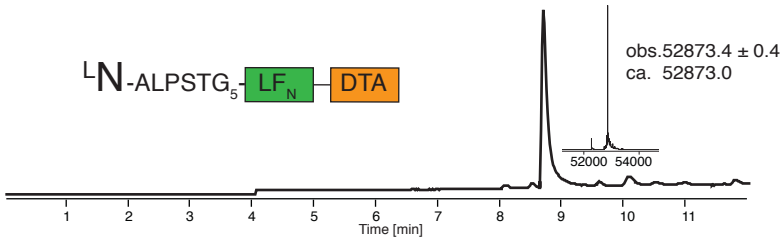

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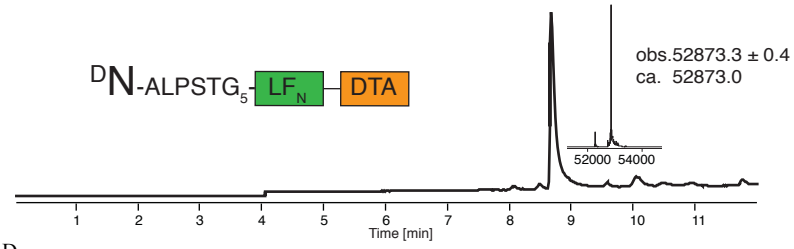

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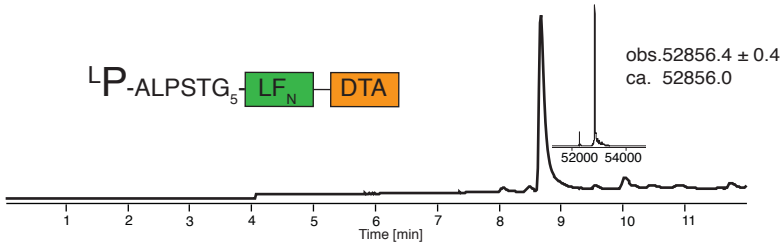

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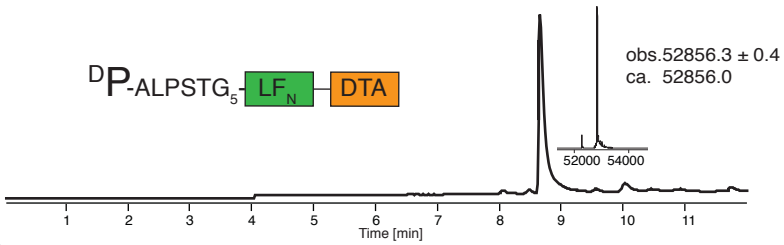

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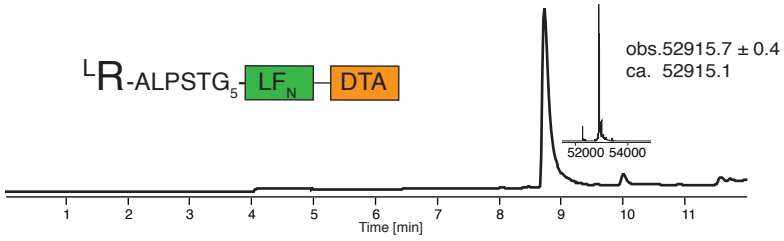

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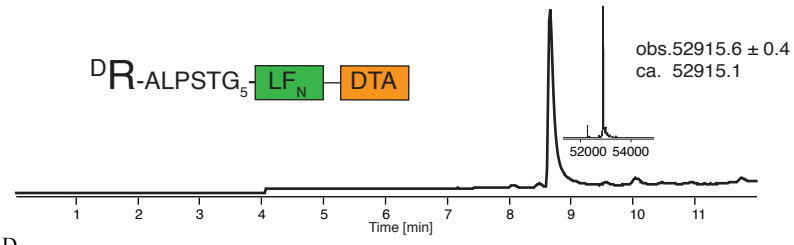

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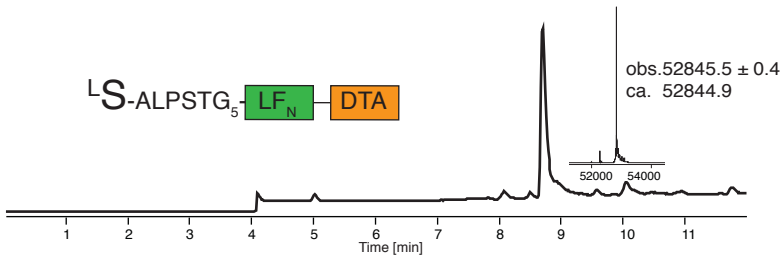

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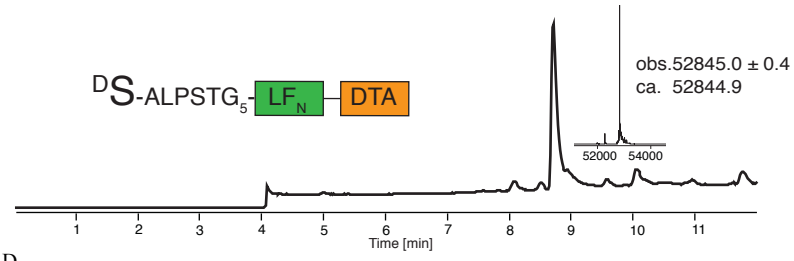

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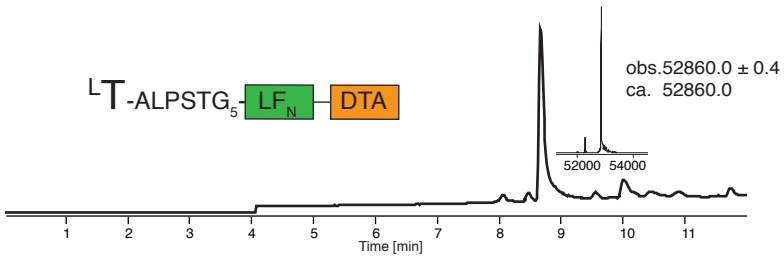

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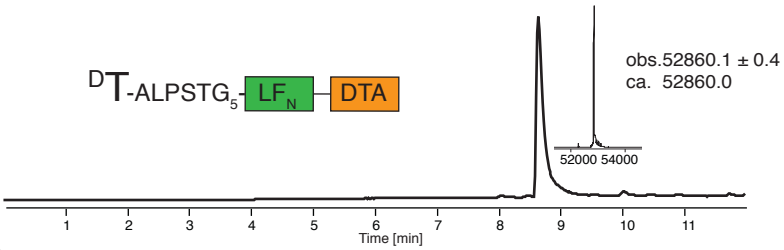

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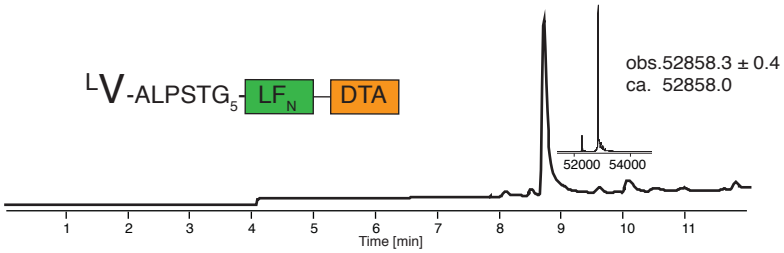

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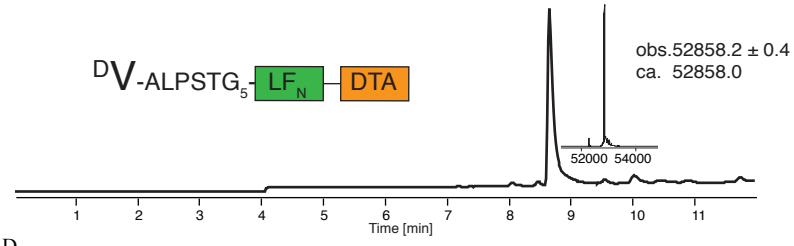

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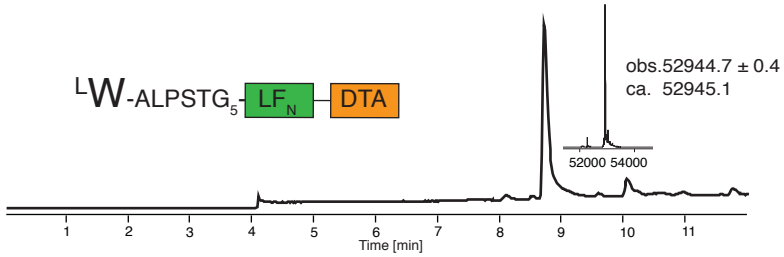

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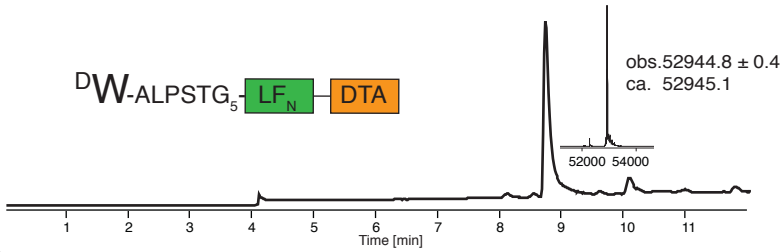

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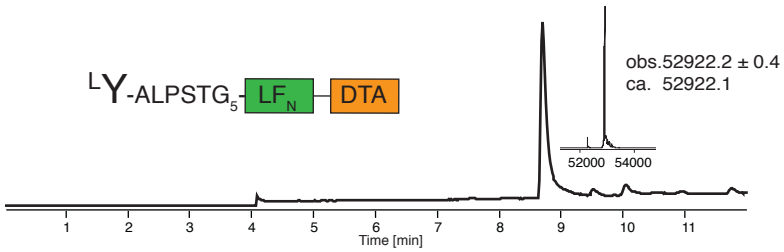

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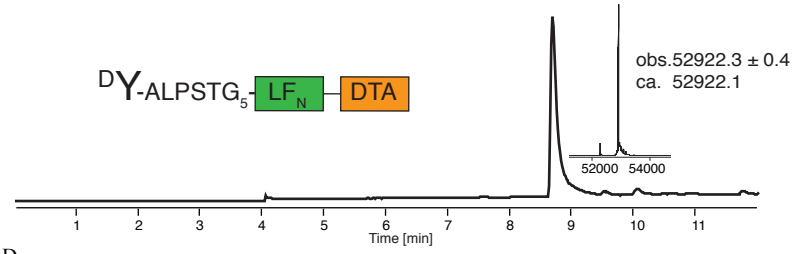

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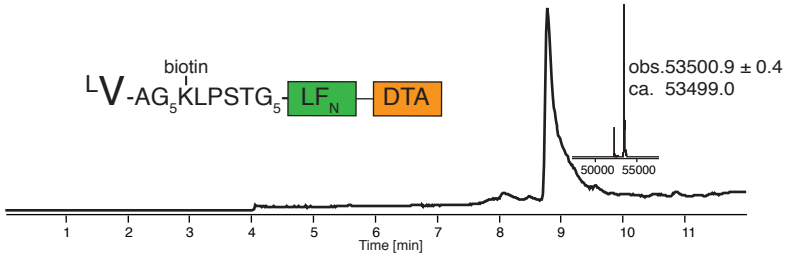

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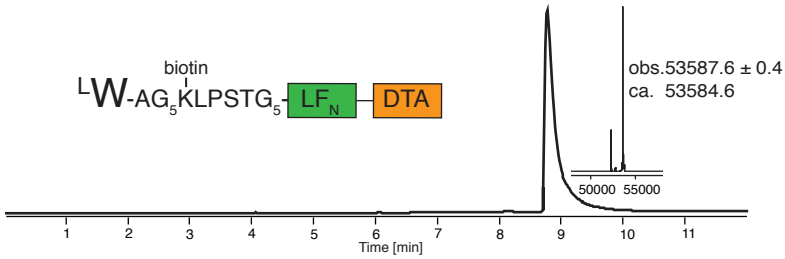
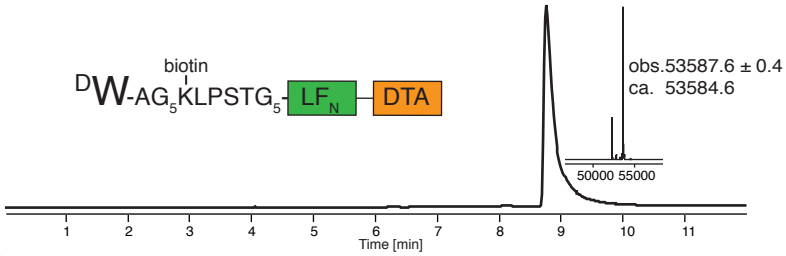


^{L}W- K(bio)-LF$_{N}$-DTA



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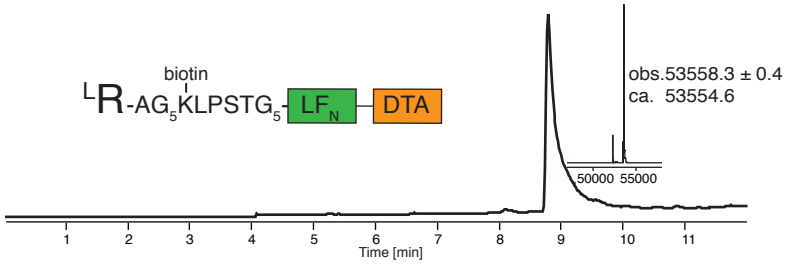
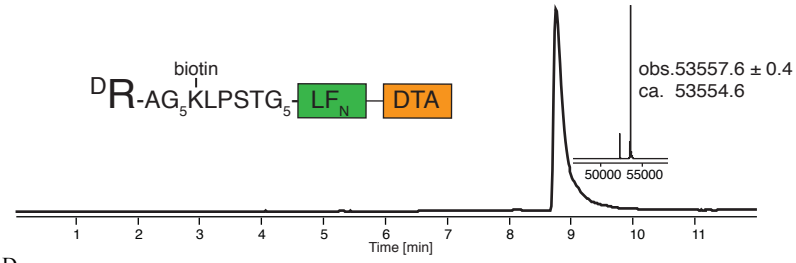


^{L}R-K(bio)-LF$_N$-DTA



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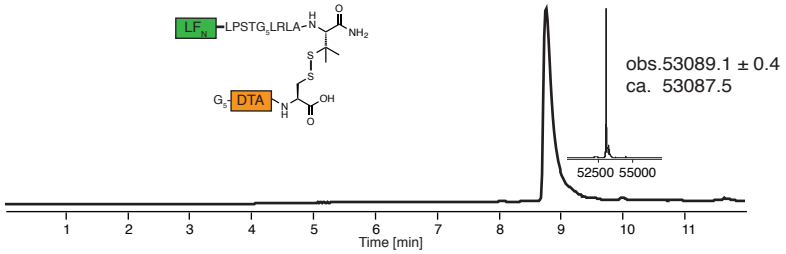
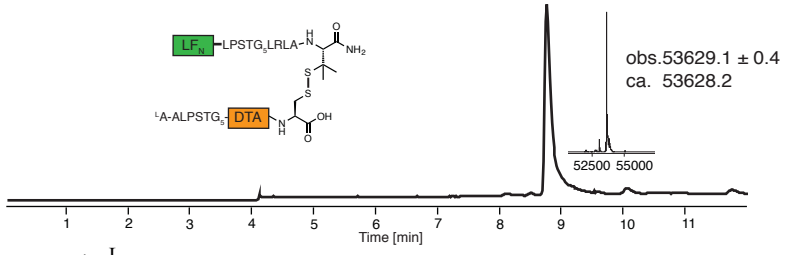
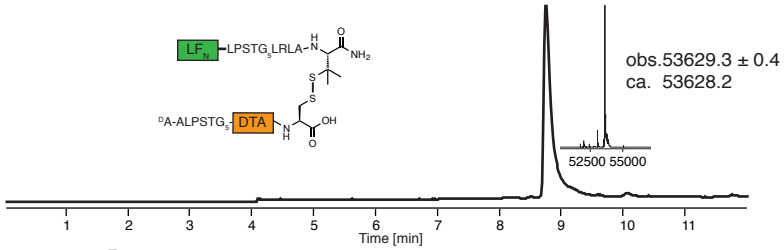


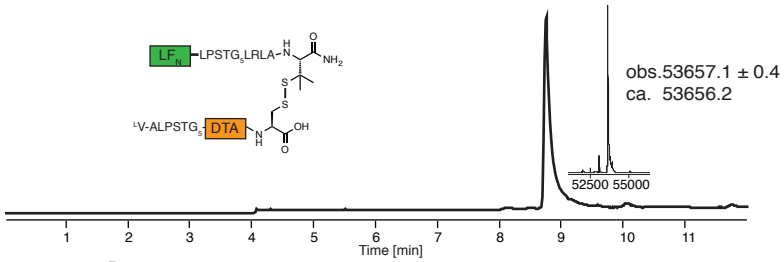
LF$_N$-C*_G$_5$-DTA-C



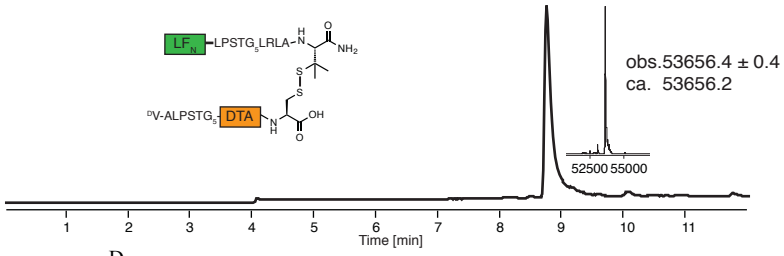
LF$_N$-C*_^{L}A-DTA-C



LF$_N$-C*_^{D}A-DTA-C



LF$_N$-C*_^{L}V-DTA-C



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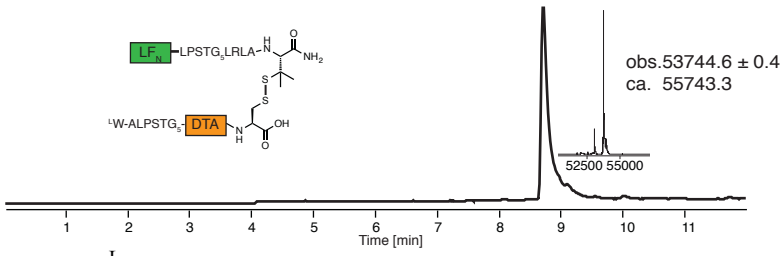

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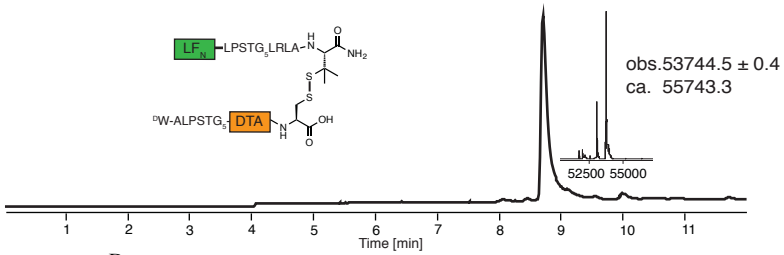
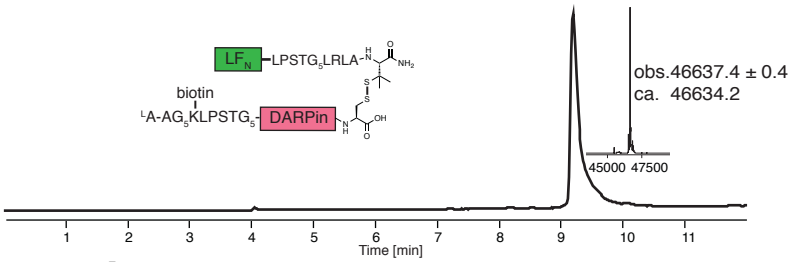
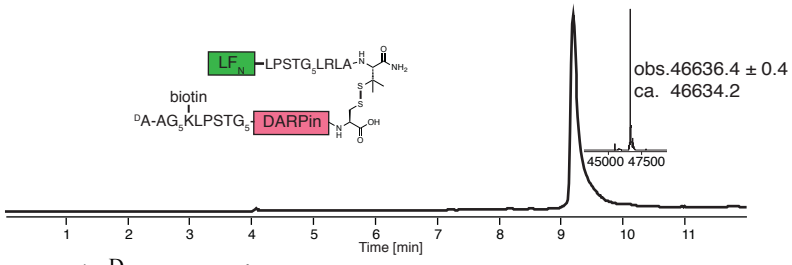


LF$_N$-C*_^{D}W-DTA-C



LF_N-C*_^{L}A-DARPin-C



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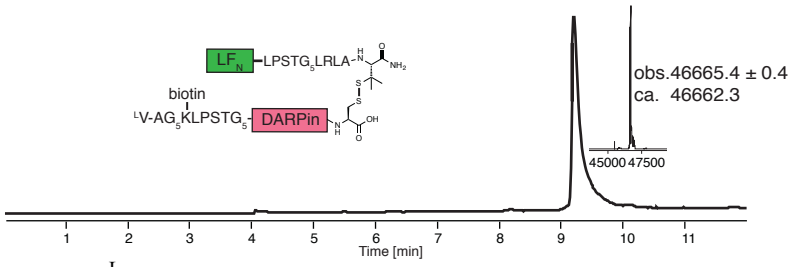

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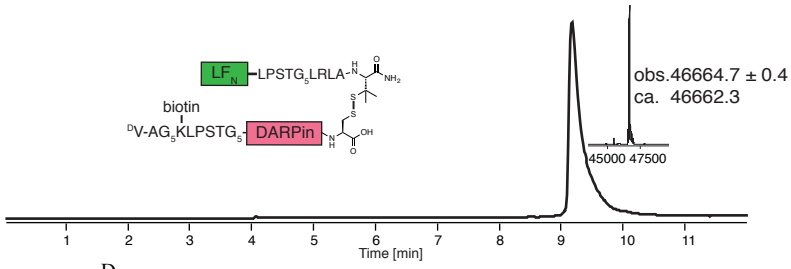

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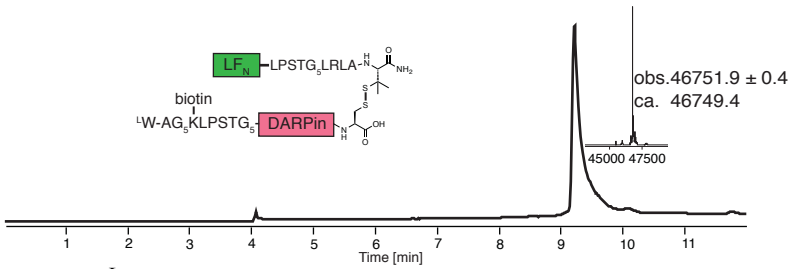
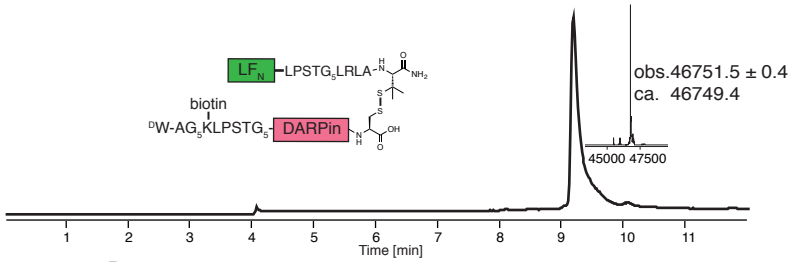


LF_N-C*_^{L}W-DARPin-C



LF$_N$-C*_^{D}W-DARPin-C

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1. Simon, M. D., Heider, P. L., Adamo, A., Vinogradov, A. A., Mong, S. K., Li, X. Y., Berger, T., Policarpo, R. L., Zhang, C., Zou, Y. K., Liao, X. L., Spokoyny, A. M., Jensen, K. F., and Pentelute, B. L. (2014) Rapid flow-based peptide synthesis, *ChemBioChem 15*, 713-720.
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3. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. (1994) Synthesis of proteins by native chemical ligation, *Science 266*, 776-779.
4. Pentelute, B. L., Barker, A. P., Janowiak, B. E., Kent, S. B. H., and Collier, R. J. (2010) A Semisynthesis Platform for Investigating Structure-Function Relationships in the N-Terminal Domain of the Anthrax Lethal Factor, *Acs Chemical Biology 5*, 359-364.