Electronic Supplementary Information

Chemical synthesis of Shiga toxin subunit B using a next-generation traceless "helping hand" solubilizing tag

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Section I: Materials

N-Fmoc-amido-dPEG®2-acid was purchased from Quanta Biosciences. Fmoc-6-aminohexanoic acid, Fmoc-7-aminoheptanoic acid, and Fmoc-8-aminocaprylic acid were purchased from Chem-Impex International. 2-chlorotrityl resin and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxidhexafluorophosphate (HATU) were purchased from ChemPep. TentaGel® R RAM resin was purchased from Rapp Polymere. Fmoc-L-Thr(tBu)-OH, Fmoc-Pro-OH, Fmoc-L-Met-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Asp(tBu)-OH, Fmoc-L-Glu(OtBu)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Trp(Boc)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Ile-OH, Fmoc-L-Val-OH, Fmoc-L-Phe-OH, Fmoc-L-Leu-OH, Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH, Fmoc-Phe-Thr($\Psi^{Me,Me}$pro)-OH, Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH, and Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH were purchased from Gyros Protein Technologies and CBL Biopharma. Fmoc-Lys(Dde)-OH was purchased from AAPPTec. Boc-L-Thr(tBu)-OH was purchased from Bachem. Dimedone, synthesis grade trifluoracetic acid (TFA), ACS grade dimethylformamide (DMF), peptide synthesis grade n-methylmorpholine (NMM), synthesis grade n-methylpyrrolidinone (NMP), ACS grade anhydrous diethyl ether, ACS grade ethyl acetate, ACS grade hexanes, HPLC grade acetonitrile (ACN), HPLC grade methanol, LC-MS grade ACN with 0.1% formic acid, and LC-MS grade water with 0.1% formic acid were

purchased from Fisher Scientific (all reagent brands from Fisher Scientific were Fisher Chemical). Piperidine, triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), 5(6)-carboxyfluorescein, N,N′-Diisopropylcarbodiimide, Oxyma Pure, anhydrous hydrazine, hydrazine monochloride, hydroxylamine monochloride, acetic anhydride, silica, 4-mercaptophenylacetic acid (MPAA), and recombinant StxB were purchased from Sigma Aldrich.

Section II: General Methods for Peptide Synthesis

Peptides were synthesized on a Prelude X instrument (Gyros Protein Technologies) using Fmoc solid-phase peptide synthesis at 30 μmol scale. Deprotection cycles employed three treatments of 2 mL 20% piperidine in DMF for 3 min followed by three washes for 30 s using 2 mL DMF. Coupling cycles consisted of addition of 0.65 mL 200 mM amino acid in NMP, 0.65 mL 195 mM HATU in DMF, and 0.5 mL 600 mM NMM in DMF. Resin and coupling reagents were then mixed using nitrogen for 25 min at room temperature before being washed three times with 2 mL DMF.

Tentagel R RAM resin (loading density 0.19 mmol/g) was utilized for the synthesis of C-terminal amides. In the case of peptide acids, 0.03 mmol Fmoc-AA was dissolved in 1 mL DMF/DCM, and then 0.15 mmol DIPEA was added to the Fmoc-AA solution. This solution was added to 150 mg of 2-cholorotrityl chloride resin and mixed for 1 h on rotisserie at room temperature. Unreacted groups were capped with 17:2:1 DCM: MeOH: DIPEA for 5 min. 2-

chlorotrityl chloride resin was converted to 2-chlorotrityl hydrazine according to published protocol to generate C-terminal hydrazides.[1] 2-chlorotrityl hydrazine resin was loaded with the first residue by dissolving 0.03 mmol Fmoc-AA and 0.03 mmol Oxyma pure in 1.2 mL DMF/DCM, followed by addition of 0.06 mmol DIC and activation for 10 min. After activation, this solution was added to 150 mg of 2-chlorotrityl hydrazine resin for 1 h on rotisserie at room temperature. Unreacted groups were capped through the addition of 1 mL of acetic anhydride and 1 mL of 0.6 M NMM for 15 min. The Fmoc-AA-resin was then washed 6x with DMF and transferred to the Prelude X for automated synthesis.

N-terminal capping was achieved through the addition of 1 mL acetic anhydride and 1 mL 0.6 M NMM per 30 μmol resin. Labeled peptides were generated using 5-(6)-carboxyfluorescein with standard coupling conditions. After completion of syntheses, peptide-resins were thoroughly washed with DCM and dried under vacuum. Cleavage of peptide resins was achieved by 180 min agitation with 4 mL TFA containing 2.5% water and 2.5% TIS per 30 μmol peptide resin. For peptides containing Cys, 2.5% EDT was added to the cleavage cocktail. For peptides containing Met, 2.5% EDT, as well as 25 mg NH_4I per mL TFA, was used. The TFA solution was precipitated into 40 mL ice-cold diethyl ether per 30 μmol crude peptide and centrifuged at 5,000 g for 10 min. The supernatant was decanted while pellets were washed twice with ether before being dried under vacuum.

Section III: Preparative HPLC Purification Methods

Crude peptide was dissolved in 20% ACN 0.1% TFA and sonicated for 5 min before centrifugation at 5,000 g (10 min) to remove undissolved material. Supernatant was filtered using a 0.2 μm nylon filter before injection. Purification was performed on an Agilent 1260 Infinity II LC system, Agilent 1260 Infinity II preparative system, or Beckman Gold 126 HPLC. Mobile phases for purification were 0.1% TFA in water (Buffer A) and 0.1% TFA in 90% ACN (Buffer B). Purification methods are described in more detail below:

Purification Method A: Phenomenex Kinetix 5 μm C18 (100 Å, 10 × 250 mm), 50 °C gradient; 0-4 min 10% B, 4-5 min 10-35% B, 5-30 min 35-45% B, 30-31 min 45-90% B, 31-36 min, 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 5 mL/min

Purification Method B: Phenomenex Kinetix 5 μm C18 (100 Å, 10 × 250 mm), 50 °C gradient; 0-4 min 20% B, 4-5 min 20-35% B, 5-30 min 35-55% B, 30-31 min 55-90% B, 31-36 min, 90% B, 36-37 min 90-20% B, 37-40 min 20% B; flow rate: 5 mL/min

Purification Method C: Phenomenex Kinetix 5 μm C18 (100 Å, 10 × 250 mm), 50 °C gradient; 0-4 min 10% B, 4-5 min 10-20% B, 5-30 min 20-44% B, 30-31 min 44-90% B, 31-36 min, 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 5 mL/min

Purification Method D: Phenomenex Jupiter 5 μm C12 (90 Å, 21.2 × 250 mm), gradient; 0-20 min 20-70% B, 20 min 100% B, 20-22 min 100% B, 22 min 20% B, 22-25 min 20% B; flow rate: 20 mL/min

Purification Method E: Phenomenex Jupiter 5 μm C4 (300 Å, 10 × 250 mm), 50 °C gradient; 0-5 min 20% B, 5 min 20-30% B, 5-30 min 30-48% B, 30-31 min 48-90% B, 31-36 min 90% B, 36-37 min 90-20% B, 37-40 min 20% B; flow rate: 6 mL/min

Section IV: Analytical HPLC/LC-MS Methods

Generally, analytical traces were collected on an Agilent 1260 Infinity II using 0.1% TFA in water (Buffer A) and 0.1% TFA in 90% ACN (Buffer B). All traces were collected at A_{214} using a single-wavelength detector. Analytical methods are described in more detail below:

Analytical Method A: Phenomenex Kinetix 5 μm C18 (100 Å, 4.6 × 150 mm), 50 °C, gradient; 0-2 min 20% B, 2-25 min 20-70% B, 25.1 min 90% B, 25.1-26 min 90% B, 26.1 min 20% B, 26.1-30 min 20% B; flow rate: 1 mL/min

Analytical Method B: Phenomenex bioZen 3.6 μm C4 (200 Å, 4.6 × 150 mm), 45 °C, gradient; 0-1 min 10% B, 1-30 min 10-60% B, 30.1 min 90% B, 30.1-32 min 90% B, 32.1 min 10% B, 32.1-35 min 10% B; flow rate: 1 mL/min

For LC-MS analysis, mobile phases were 0.1% FA in water (Buffer A) and 0.1% FA in ACN (Buffer B). Mass spectra were collected on an Agilent 6120 single-quadrupole mass spectrometer in fast scan/positive ion mode with an Agilent 1260 Infinity II front-end. UV data were collected using the Agilent 1260 Infinity II diode array detector (200-600 nm). Observed masses were determined using Agilent Chemstation's deconvolution algorithm with averaged

scans across the major ion signal and corresponding UV peak. Calculated and observed masses are presented as average mass. LC-MS methods are described in more detail below:

LC-MS Method A: Agilent Poroshell 2.7 μm EC-C18 (120 Å, 4.6 × 50 mm), 50 °C, gradient: 0-1 min 5% B, 1-8 min 5-90% B, 8.1-10 min 5% B, flow rate: 0-8 min 0.75 mL/min, 8.1-10 min 1.0 mL/min; scan range: 400-2,000 *m/z*

LC-MS Method B: Agilent Poroshell 2.7 μm EC-C18 (120 Å, 4.6 × 50 mm), 50 °C, gradient: 0-1 min 20% B, 1-11 min 20-50% B, 11.1-12 min 90% B, 12.1-13 min 5% B flow rate: 1.0 mL/min; scan range: 400-2,000 *m/z*

LC-MS Method C: Agilent Poroshell 2.7 μm EC-C18 (120 Å, 4.6 × 50 mm), 50 °C, gradient; 0-1 min 5% B, 1.1-10 min 10-90% B, 11 min 90% B, 11.1-13 min 5% B; flow rate: 0-10 min 0.75 mL/min, 11-13 min 1.0 mL/min; scan range: 50-750 *m/z*

LC-MS Method D: Agilent Poroshell 2.7 μm EC-C18 (120 Å, 4.6 × 50 mm), 50 °C, gradient; 0-1 min 5-15% B, 1-12 min 15-60% B, 12.1 min 90% B, 12.1-13 min 90% B, 13.1 5% B; flow rate: 1 mL/min; scan range: 400-2000 *m/z*

Section V: Synthesis and Characterization of Fmoc-Ddae

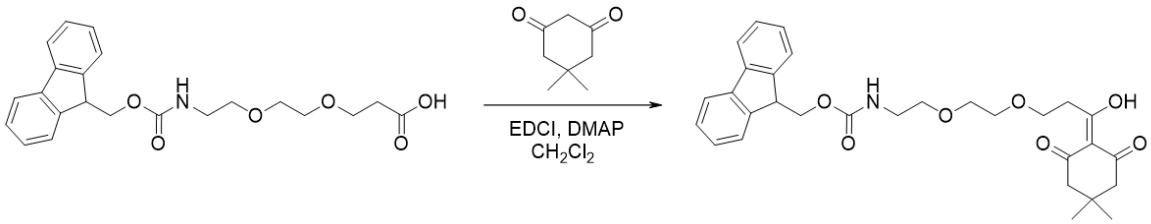

Figure S1: One-step synthesis of *N*-Fmoc-1-(4,4-dimethyl-2,6-dioxocyclo-hexylidene)-3-[2-(2-aminoethoxy)ethoxy]-propan-1-ol, (Fmoc-Ddae-OH)

Following previously published protocol,[2] a solution of *N*-Fmoc-amido-dPEG®$_2$-acid (0.5 g, 1.25 mmol), dimedone (228 mg, 1.3 equiv.) and EDC·HCl (252 mg, 1.05 equiv.) in CH_2Cl_2 (10 mL) was cooled to 0°C before addition of DMAP (199 mg, 1.3 equiv.). The solution was allowed to warm and stirred at room temperature for 5 h. The mixture was diluted with AcOEt (60 mL) and washed with 1 M aqueous HCl (3 × 20 mL), 5% $NaHCO_3$ (3 × 20 mL), then saturated aqueous NaCl (20 mL). The organic layer was dried over $MgSO_4$, filtered, and then concentrated under reduced pressure. Purification by flash column chromatography (eluent: petroleum ether/AcOEt 3:1 then 1:1 then 4:6) afforded pure Fmoc-Ddae-OH as a thick oil (365 mg, 50%).

^{1}H NMR (600 MHz, $CDCl_3$): δ = 17.93 (s, 1H, OH), 7.73 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.58 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.38 (t, *J* = 7.4 Hz, 2H, H_{Ar}), 7.27 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 5.51 (bt, *J* = 5.7 Hz, 1H, NH), 4.36 (d, *J* = 7.2 Hz, 2H, CH_{Fmoc}), 4.21 (t, *J* = 7.2 Hz, 1H, $CH_{2\ Fmoc}$), 3.82 (t, *J* = 6.2 Hz, 2H, CH_2N), 3.49-3.56 (m, 4H, CH_2O), 3.47 (bt, *J* = 5.1 Hz, 2H, CH_2O), 3.28-3.38 (m, 2H, CH_2O), 3.26 (t, *J* = 5.8 Hz, 2H, CH_2COH), 2.47 (s, 2H, CH_2CO), 2.29 (s, 2H, CH_2CO), 1.02 (s, 6H, Me).

^{13}C NMR (151 MHz, $CDCl_3$): δ = 203.1, 197.1, 195.2, 156.6, 144.1, 141.3, 127.7, 127.1, 125.2, 120.0, 112.3, 70.3, 70.2, 70.1, 66.7, 66.1, 52.5, 47.3, 46.4, 41.0, 40.9, 30.7, 28.2.

LC-MS: Acquired using **LC-MS Method C**. t_R = 9.4 min, (*m/z*): $[M+H]^{1+}$ calculated for $C_{30}H_{36}NO_7$: 522.25, found: 522.2

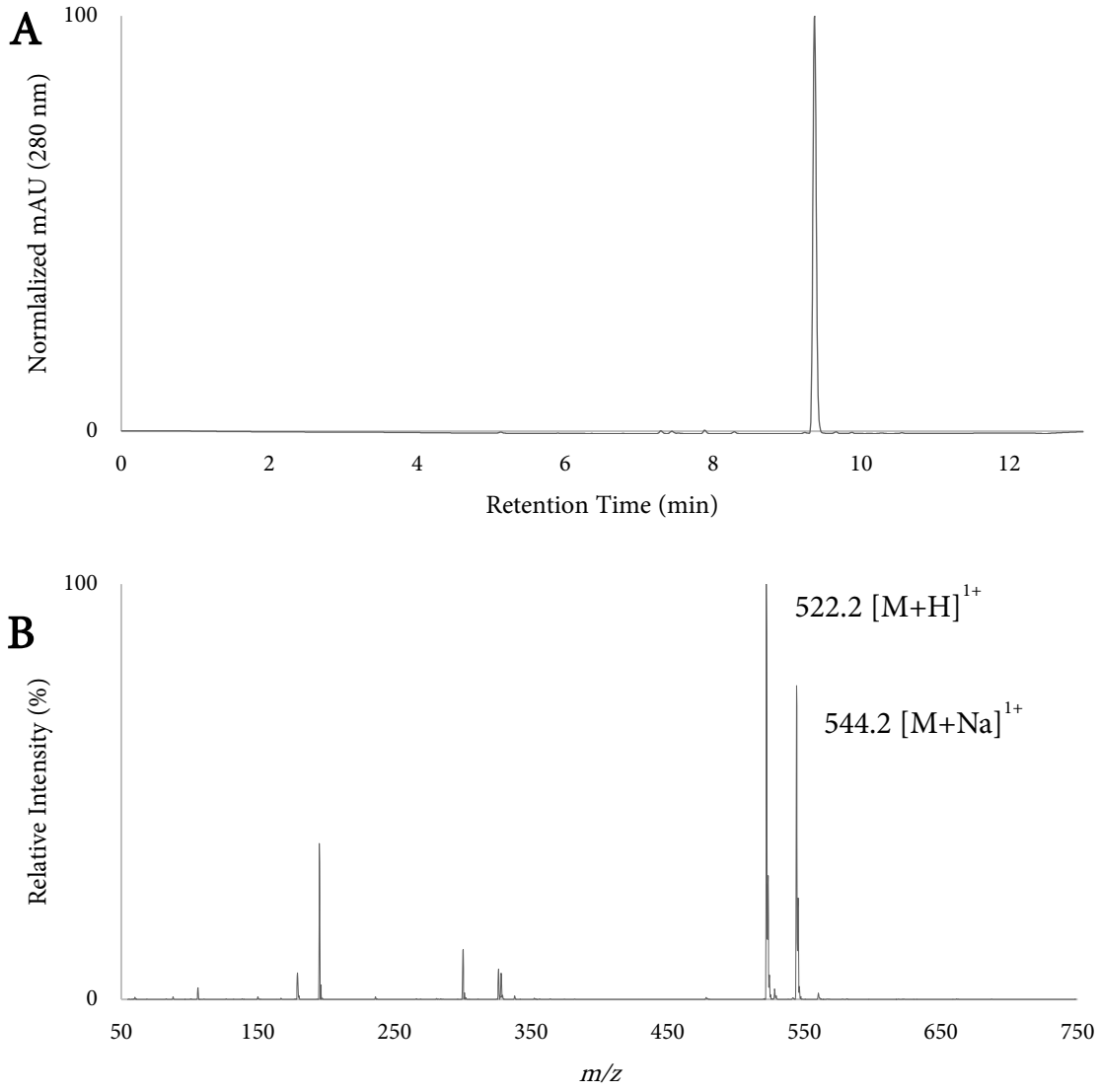

Figure S2: (A) HPLC analysis of purified Fmoc-Ddae-OH **(B)** MS analysis of purified Fmoc-Ddae-OH

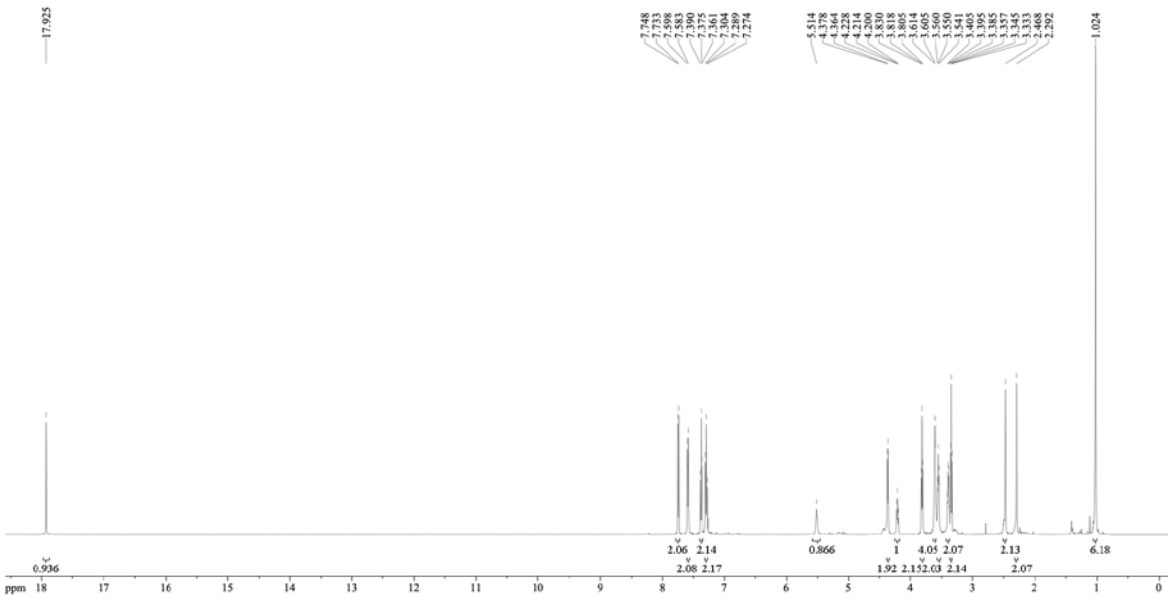

Figure S3: ^{1}H NMR of purified Fmoc-Ddae-OH

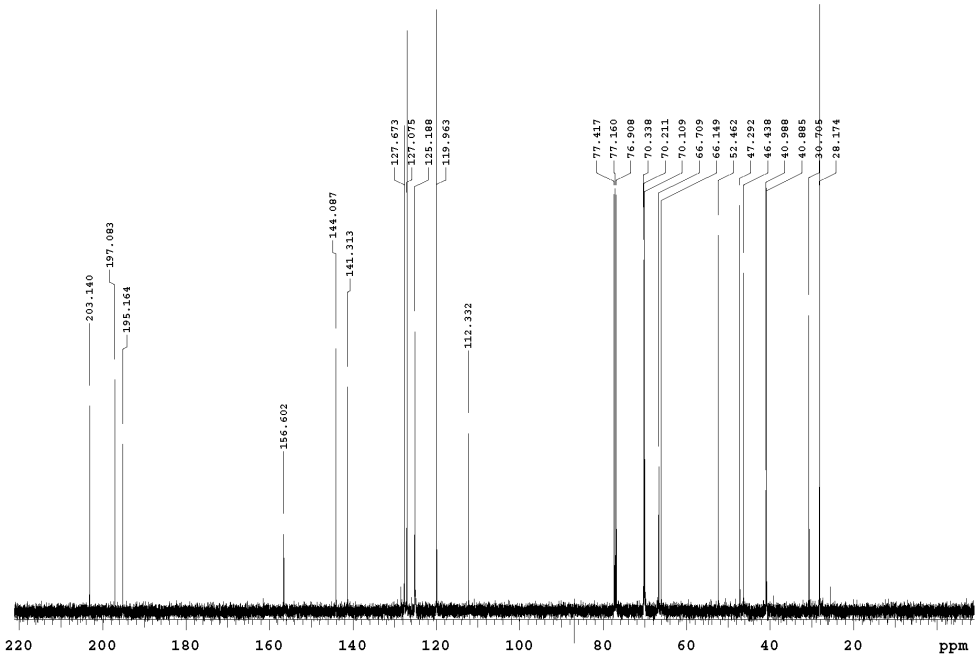

Figure S4: ^{13}C NMR of purified Fmoc-Ddae-OH

Section VI: Synthesis and Characterization of Fmoc-Ddax

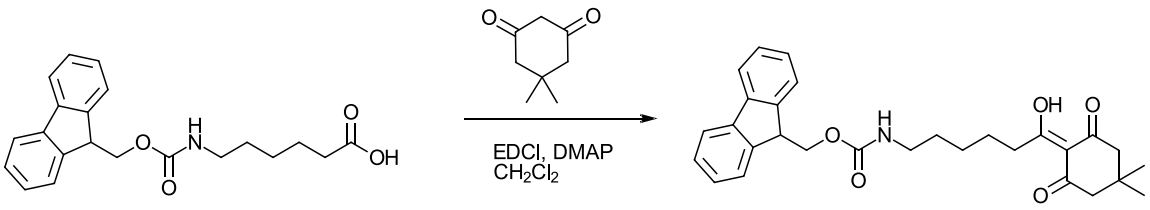

Figure S5: One-step synthesis of *N*-Fmoc-2-(6-amino-1-hydroxyhexylidene)-5,5-dimethylcyclohexane-1,3-dione, (Fmoc-Ddax-OH)

A solution of Fmoc-6-aminohexanoic acid (1.060 g, 3 mmol), dimedone (547 mg, 1.3 equiv.) and EDC·HCl (604 mg, 1.05 equiv.) in CH_2Cl_2 (10 mL) was cooled to 0°C, before addition of DMAP (476 mg, 1.3 equiv.). The solution was allowed to warm and stirred at room temperature for 5 h. The mixture was diluted with AcOEt (60 mL) and washed with 1 M aqueous HCl (3 × 20 mL), 5% aqueous $NaHCO_3$ (3 × 20 mL), then saturated aqueous NaCl (20 mL). The organic layer was dried over $MgSO_4$, filtered, and then concentrated under reduced pressure. Purification by flash column chromatography (eluent: hexanes/AcOEt 9:1 then 3:1 then 1:1) afforded pure Fmoc-Ddax-OH as a thick oil (780mg, 49%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 18.18 (s, 1H, OH), 7.72 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.56 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.36 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 7.28 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 4.92 (bs, 1H, NH), 4.36 (d, *J* = 7.0 Hz, 2H, $CH_{2\ Fmoc}$), 4.18 (t, *J* = 7.0 Hz, 1H, CH_{Fmoc}), 3.17 (q, *J* = 6.5 Hz, 2H, CH_2N), 3.00 (t, *J* = 7.5 Hz, 2H, CH_2COH), 2.49 (s, 2H, CH_2CO), 2.31 (s, 2H, CH2CO), 1.62 (quint, *J* = 7.0, 2H, CH_2CH_2NH), 1.51 (quint, *J* = 7.0, 2H, CH_2CH_2CO), 1.41-1.34 (m 2H, CH_2), 1.03 (s, 6H, Me)

^{13}C NMR (125 MHz, $CDCl_3$): δ = 205.3, 197.8, 195.2, 156.5, 144.1, 141.3, 127.7, 127.1, 125.1, 120.0, 112.0, 66.5, 52.6, 47.3, 46.8, 40.9, 40.2, 30.7, 29.7, 28.2, 26.5, 24.2.

LC-MS: Acquired using **LC-MS Method C**. t_R= 10.0 min, (*m/z*): $[M+H]^{1+}$ calculated for $C_{29}H_{33}NO_5$: 476.24, found: 476.2

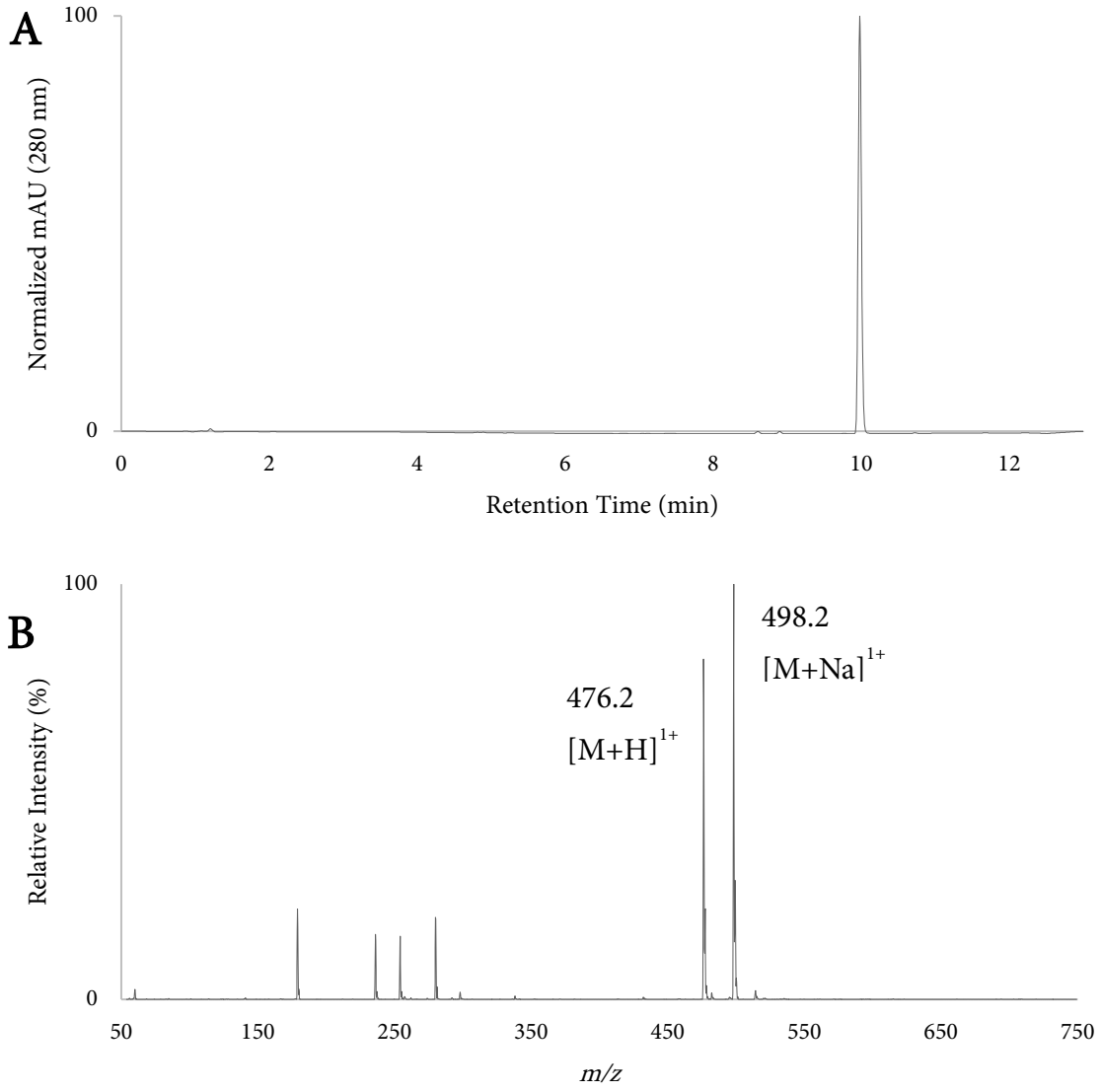

Figure S6: (A) HPLC analysis of purified Fmoc-Ddax-OH **(B)** MS analysis of purified Fmoc-Ddax-OH

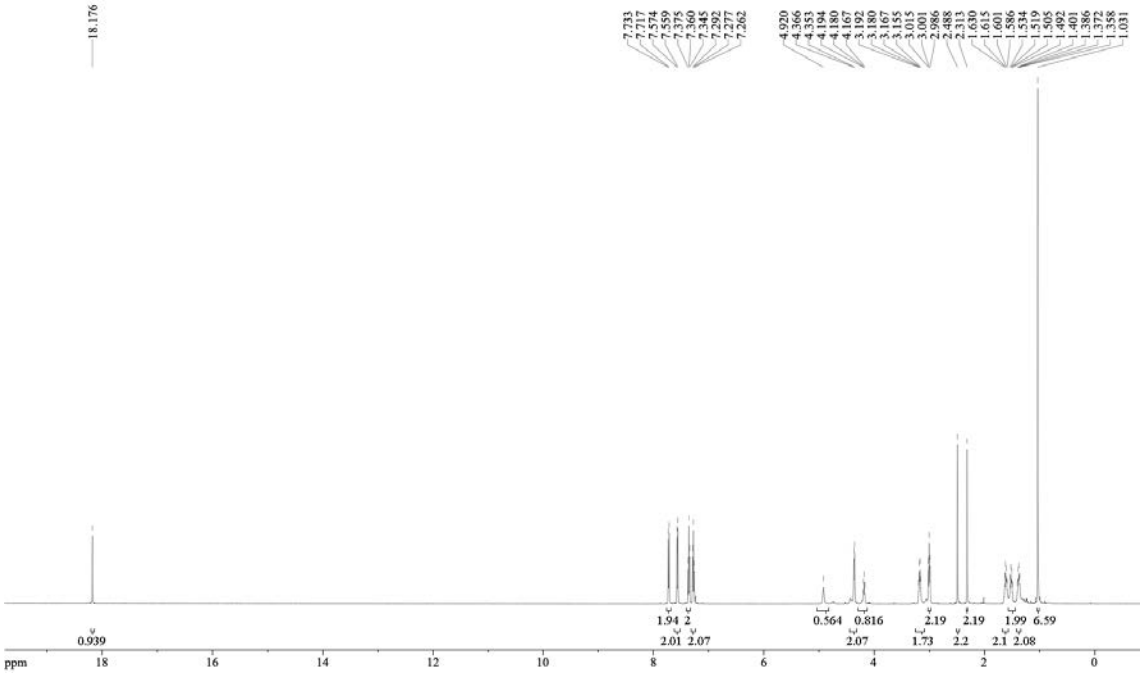

Figure S7: ^{1}H NMR of purified Fmoc-Ddax-OH

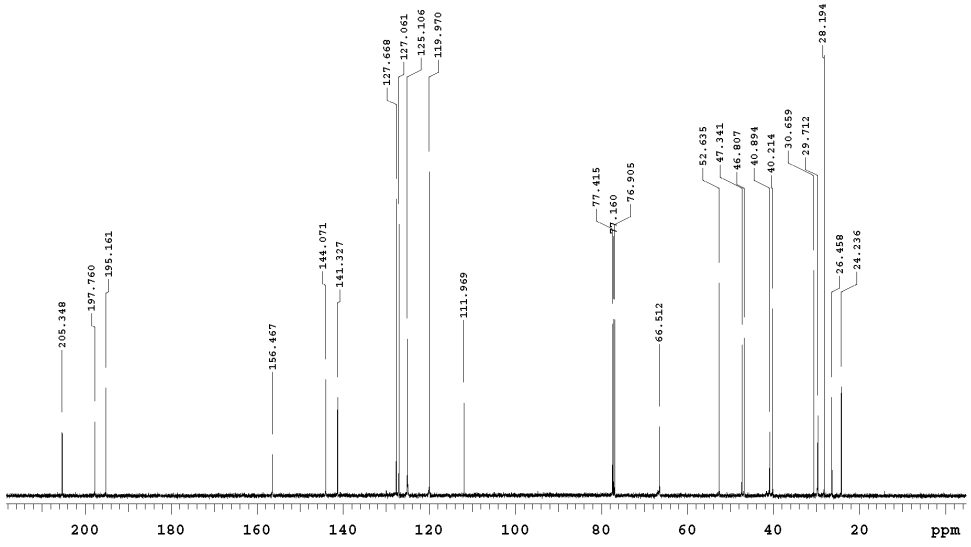

Figure S8: ^{13}C NMR of purified Fmoc-Ddax-OH

Section VII: Synthesis and Characterization of Fmoc-Ddap

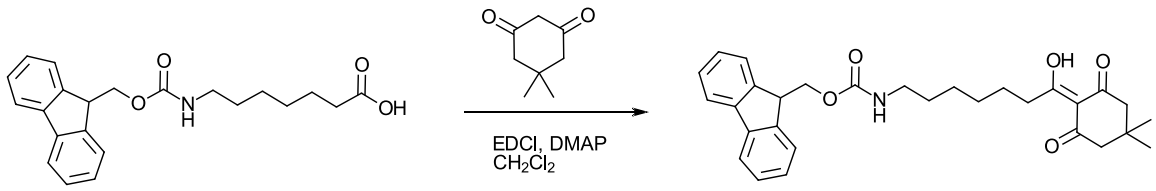

Figure S9: One-step synthesis of *N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione, (Fmoc-Ddap-OH)

A solution of Fmoc-7-aminoheptanoic acid (1.013 g, 2.7 mmol), dimedone (496 mg, 1.3 equiv.) and EDC·HCl (548 mg, 1.05 equiv.) in CH_2Cl_2 (10 mL) was cooled to 0°C, before addition of DMAP (432 mg, 1.3 equiv.). The solution was allowed to warm and stirred at room temperature for 5 h. The mixture was diluted with AcOEt (60 mL) and washed with 1 M aqueous HCl (3 × 20 mL), 5% aqueous $NaHCO_3$ (3 × 20 mL), then saturated aqueous NaCl (20 mL). The organic layer was dried over $MgSO_4$, filtered, and then concentrated under reduced pressure. Purification by flash column chromatography (eluent: hexanes/AcOEt 9:1 then 2:1) afforded pure Fmoc-Ddap-OH as a white solid (810 mg, 54%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 18.23 (s, 1H, OH), 7.74 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.58 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.38 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 7.29 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 4.92 (bs, 1H, NH), 4.37 (d, *J* = 7.0 Hz, 2H, CH_{2Fmoc}), 4.20 (t, *J* = 6.5 Hz, 1H, CH_{Fmoc}), 3.17 (bs, 2H, CH_2N), 3.01 (t, *J* = 7.0 Hz, 2H, CH_2COH), 2.51 (s, 2H, CH_2CO), 2.33 (s, 2H, CH_2CO), 1.61 (quint, *J* = 7.0, 2H, CH_2CH_2NH), 1.50 (quint, *J* = 6.5, 2H, CH_2CH_2CO), 1.42-1.32 (m 4H, CH_2), 1.06 (s, 6H, Me)

^{13}C NMR (125 MHz, $CDCl_3$): δ = 205.5, 197.8, 195.1, 156.5, 144.1, 141.3, 127.7, 127.0, 125.1, 120.0, 112.0, 66.5, 52.6, 47.3, 46.8, 41.0, 40.2, 30.6, 29.8, 29.0, 28.2, 26.5, 24.5.

LC-MS: Acquired using **LC-MS Method C**. t_R= 10.3 min, (*m/z*): $[M+H]^{1+}$ calcd for $C_{30}H_{35}NO_5$: 490.24, found: 490.2

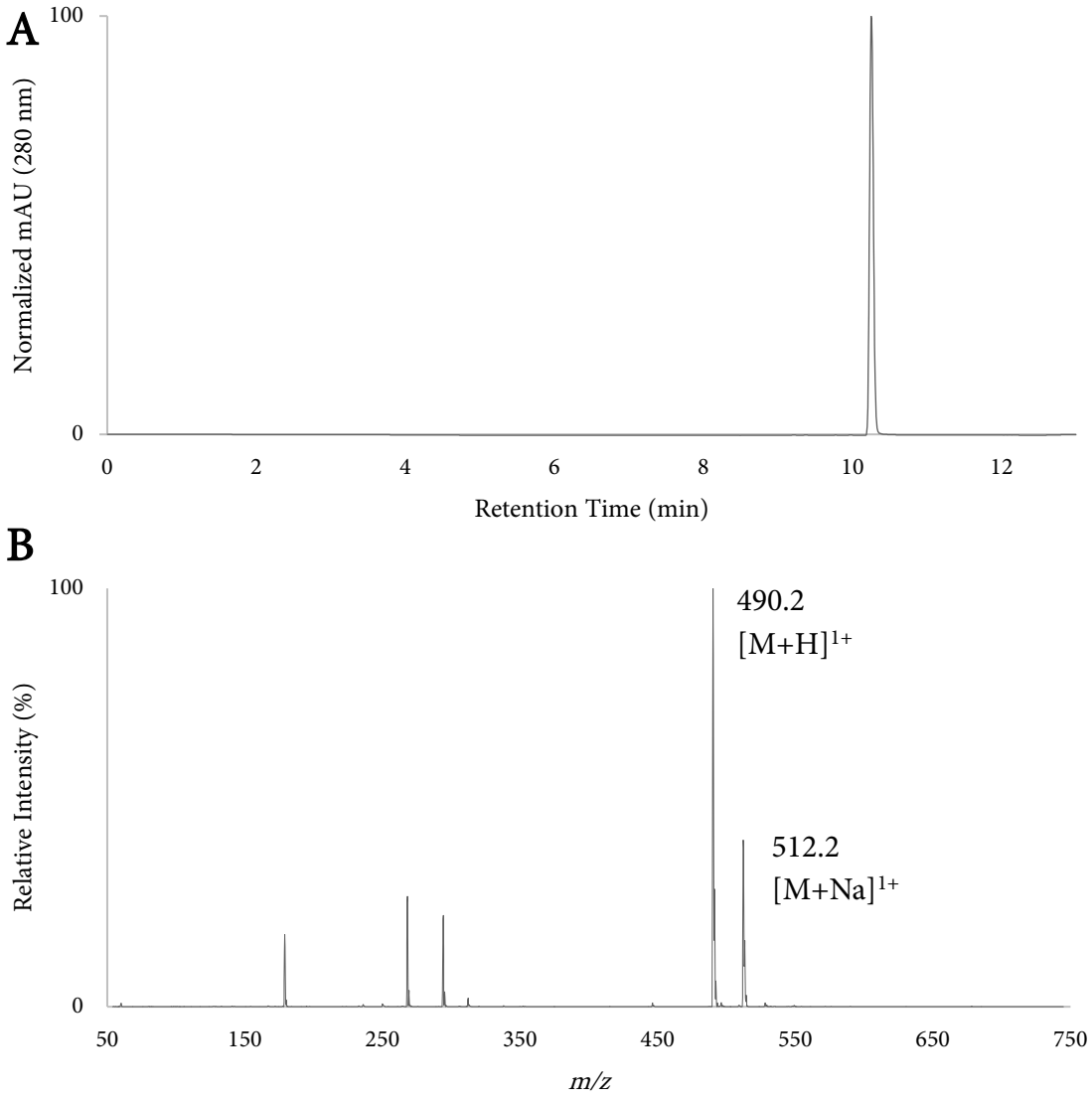

Figure S10: (A) HPLC analysis of purified Fmoc-Ddap-OH **(B)** MS analysis of purified Fmoc-Ddap-OH

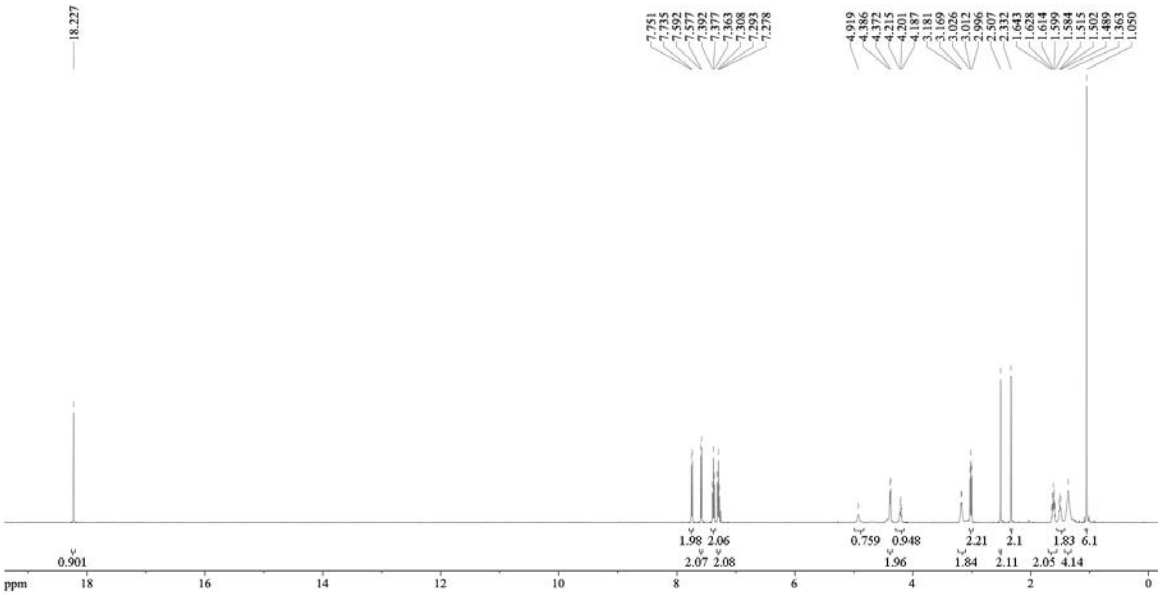

Figure S11: ^{1}H NMR of purified Fmoc-Ddap-OH

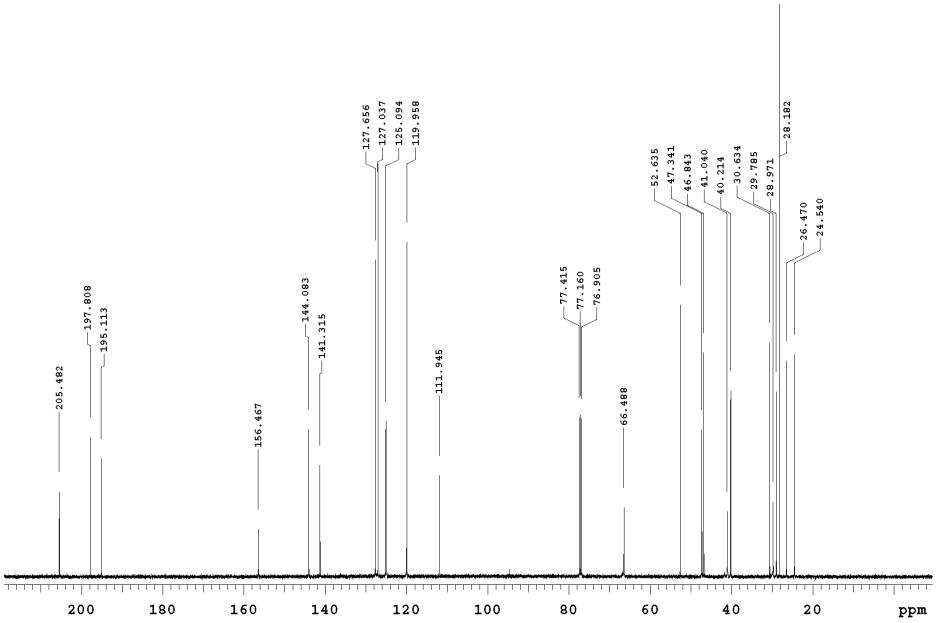

Figure S12: ^{13}C NMR of purified Fmoc-Ddap-OH

Section VIII: Synthesis and Characterization of Fmoc-Ddac

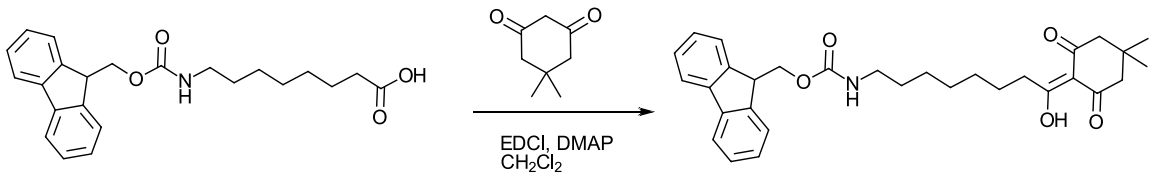

Figure S13: One-step synthesis of N-Fmoc-2-(8-amino-1-hydroxyoctylidene)-5,5-dimethylcyclohexane-1,3-dione, (Fmoc-Ddac-OH)

A solution of Fmoc-8-aminooctanoic acid (1.0 g, 2.6 mmol), dimedone (477 mg, 1.3 equiv.), and EDC·HCl (518 mg, 1.05 equiv.) in CH_2Cl_2 (10 mL) was cooled to 0°C before addition of DMAP (415 mg, 1.3 equiv.). The solution was allowed to warm and stirred at room temperature for 5 h. The mixture was diluted with AcOEt (60 mL) and washed with 1 M aqueous HCl (3 × 20 mL), 5% aqueous $NaHCO_3$ (3 × 20 mL), then saturated aqueous NaCl (20 mL). The organic layer was dried over $MgSO_4$, filtered, and then concentrated under reduced pressure. Purification by flash column chromatography (eluent: hexanes/AcOEt 9:1 then 4:1) afforded pure Fmoc-Ddac-OH as a white solid (409 mg, 28%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 18.24 (s, 1H, OH), 7.76 (d, *J* = 8.0 Hz, 2H, H_{Ar}), 7.59 (d, *J* = 7.5 Hz, 2H, H_{Ar}), 7.40 (t, *J* = 8.0 Hz, 2H, H_{Ar}), 7.31 (t, *J* = 7.5 Hz, 2H, H_{Ar}), 4.81 (bs, 1H, NH), 4.39 (d, *J* = 6.5 Hz, 2H, CH_{2Fmoc}), 4.22 (t, *J* = 6.5 Hz, 1H, CH_{Fmoc}), 3.19 (q, *J* = 6.5 Hz, 2H, CH_2N), 3.02 (t, *J* = 7.0 Hz, 2H, CH_2COH), 2.53 (s, 2H, CH_2CO), 2.35 (s, 2H, CH_2CO), 1.63-1.58 (m 4H, CH_2), 1.51 (quint, *J* = 7.0, 2H, CH_2CH_2CO), 1.42-1.28 (m 6H, CH_2), 1.07 (s, 6H, Me)

^{13}C NMR (125 MHz, $CDCl_3$): δ = 205.6, 197.9, 195.2, 156.5, 144.1, 141.4, 127.7, 127.1, 125.1,120.0,112.0,66.5,52.7,47.4,46.9,41.1,40.3,30.7, 30.0, 29.3, 29.0, 28.2, 26.6, 24.6.

LC-MS: Acquired using **LC-MS Method C.** t_R= 10.5 min, (*m/z*): $[M+H]^{1+}$ calcd for $C_{31}H_{37}NO_5$: 504.24, found: 504.2

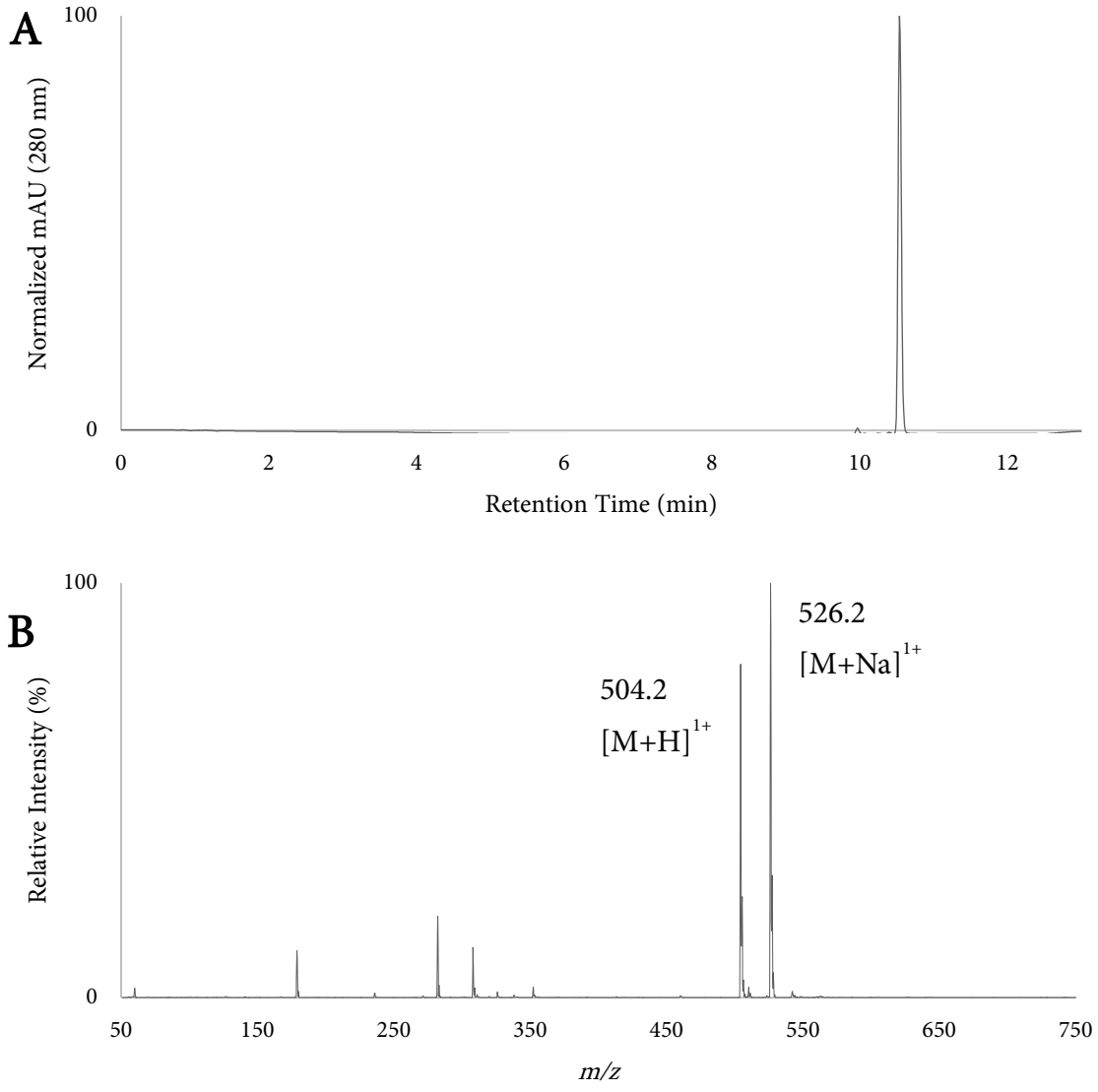

Figure S14: (A) HPLC analysis of purified Fmoc-Ddac-OH **(B)** MS analysis of purified Fmoc-Ddac-OH

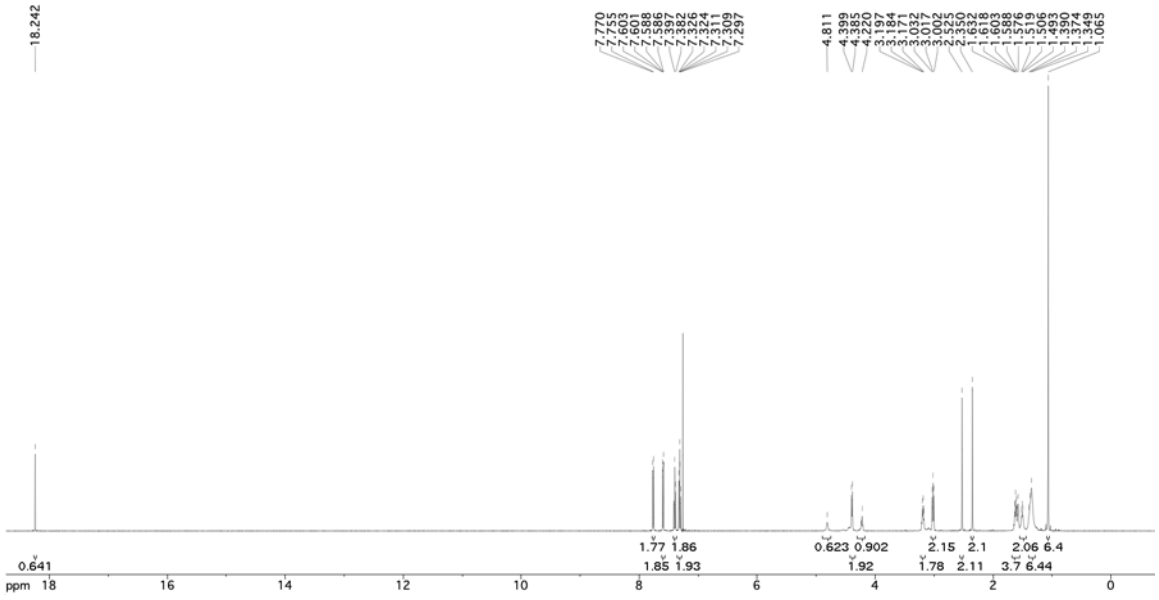

Figure S15: ^{1}H NMR of purified Fmoc-Ddac-OH

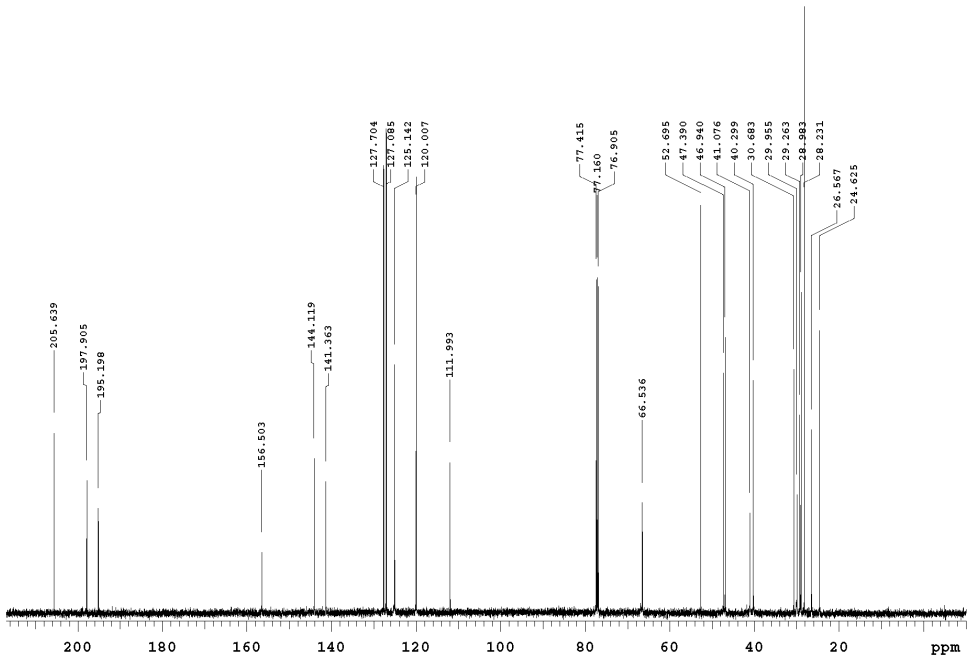

Figure S16: ^{13}C NMR of purified Fmoc-Ddac-OH

Linker	Starting Material	Supplier	Cost for 5g
Ddae	*N*-Fmoc-amido-dPEG®2-acid	Quanta Biosciences	$400
Ddax	Fmoc-6-aminohexanoic acid	Chem-Impex	$20
Ddap	Fmoc-7-aminoheptanoic acid	Chem-Impex	$110
Ddac	Fmoc-8-aminocaprylic acid	Chem-Impex	$190

Table S1: Cost comparison of each linker's starting material (as of February 2019).

Figure S17: Image comparing the physical state of each linker synthesized in this study (from left to right; ~200 μmol of Ddax, Ddap, Ddac, and Ddae)

Section IX: Synthesis of Helping Hand-Modified Peptides

Peptides to be modified with helping hands were prepared by standard Fmoc-SPPS as described above in addition to the following steps. Fmoc-Lys(Dde)-OH building block was used at the position to be modified with the linker. The N-terminus of the peptide was protected: either by capping with acetyl group in the case of C20 peptide or through the use of Boc-Thr(tBu)-OH building block in the case of StxB-N. Upon completion of the linear peptide synthesis, the Dde group was removed by 3 × 10 min treatment with 2 mL of 5% hydrazine in DMF. After washing thoroughly with DMF, the resin was treated with Fmoc-Ddae-OH, Fmoc-Ddax-OH, Fmoc-Ddap-OH, or Fmoc-Ddac-OH (1 mL, 200 mM in NMP) for >2 h at 37°C. Standard Fmoc-SPPS conditions were used to install the helping hand sequence, and standard peptide cleavage conditions (described above) were employed following installation of the helping hand onto the peptide.

Section X: Synthesis and Characterization of C20 Model Peptides

The C20 peptide (DWTKNITDKIDQIIHDFVDK) was utilized as a model peptide to characterize the properties of each linker used in this study. This peptide is derived from the C-terminal heptad repeat region of the Ebolavirus GP2 protein.[3,4] The C20 peptide was selected for several reasons: good aqueous solubility, >70% crude peptide purity, a relatively diverse peptide sequence (ten different amino acids), and previously demonstrated use as a model peptide.[2]

Characterization of the linkers using an easy-to-work with model peptide allows for a straightforward approach in data analysis and interpretation of results.

Peptide	Sequence
C20	Acetyl—DWTKNITDK(**Dde**)IDQIIHDFVDK—NH_2
C20(Lys_6-Ddae)	Acetyl—DWTKNITDK(**KKKKKK-Ddae**)IDQIIHDFVDK—NH_2
C20(Lys_6-Ddax)	Acetyl—DWTKNITDK(**KKKKKK-Ddax)**IDQIIHDFVDK—NH_2
C20(Lys_6-Ddap)	Acetyl—DWTKNITDK(**KKKKKK-Ddap**)IDQIIHDFVDK—NH_2
C20(Lys_6-Ddac)	Acetyl—DWTKNITDK(**KKKKKK-Ddac**)IDQIIHDFVDK—NH_2
Fl-C20	Fluorescein—DWTKNITDKIDQIIHDFVDK—NH_2
Fl-C20(N_3-Ddap)	Fluorescein—DWTKNITDK(**6-azido-Ddap**)IDQIIHDFVDK—NH_2

Table S2: Sequences of all C20 model peptides used in this study. Note, the Dde group on C20 was removed before analysis by LC-MS.

C20 peptides (30 μmol scale) were prepared on TentaGel R RAM resin to generate C-terminal amides. N-termini were capped using 1 mL acetic anhydride and 1 mL of 0.6 M NMM for 15 min per 30 μmol scale synthesis. Lys(Dde) was incorporated at an internal Lys in C20 (indicated in bold/underline above). The Dde group was removed by 3 × 10 min treatment with 2 mL of 5% hydrazine in DMF.

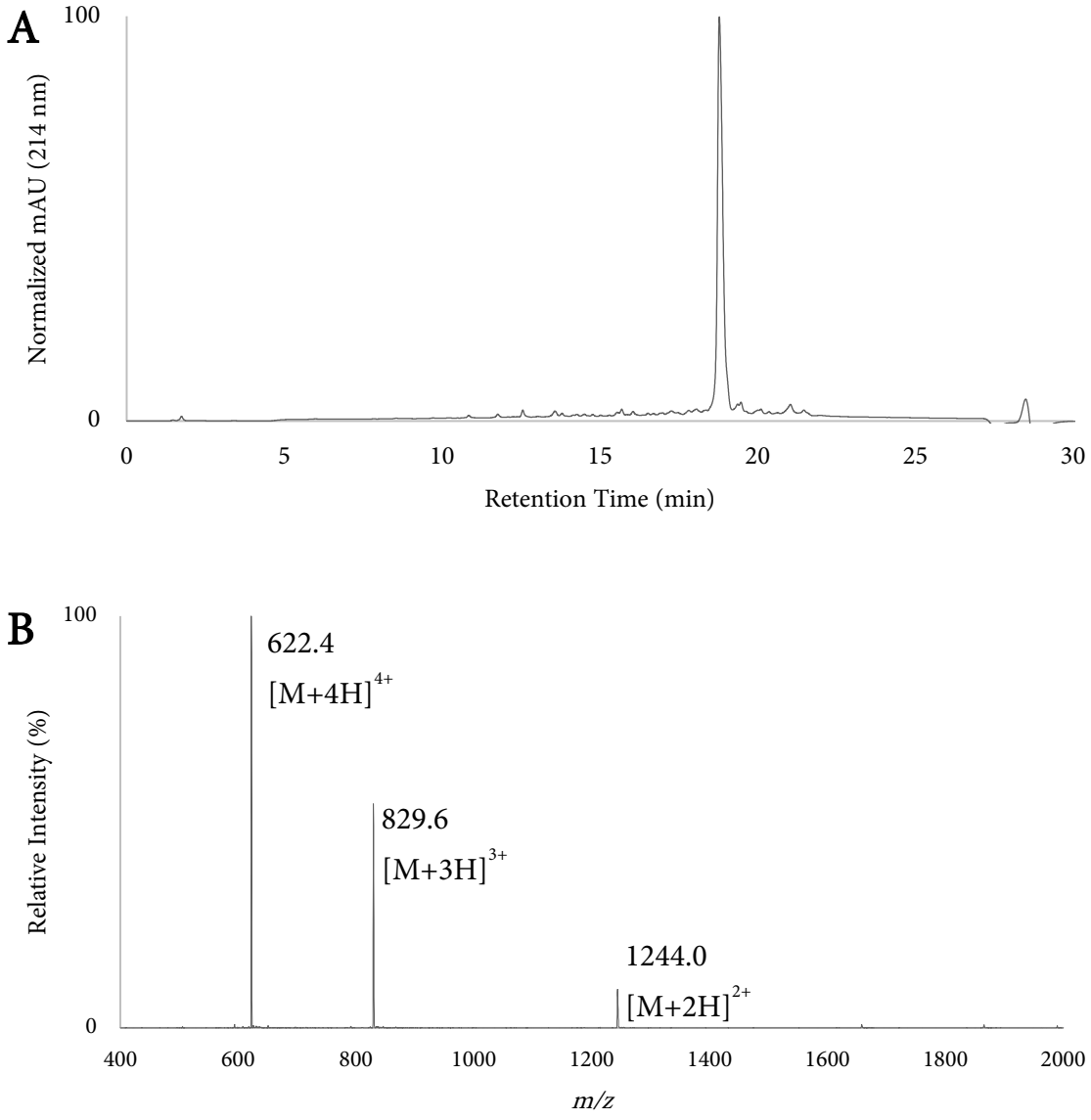

Figure S18: (A) HPLC analysis of crude C20 peptide (Ac-DWTKNITDKIDQIIHDFVDK-NH_2) using **Analytical Method A** (t_R= 17.5 min)**. (B)** Mass spectrum of crude C20 peptide determined with **LC-MS Method A**. Calculated mass = 2485.70 Da, Observed mass = 2485.7 Da

Coupling of each linker was performed with 1 mL of 200 mM linker in NMP at 37°C per 30 µmol peptide resin.

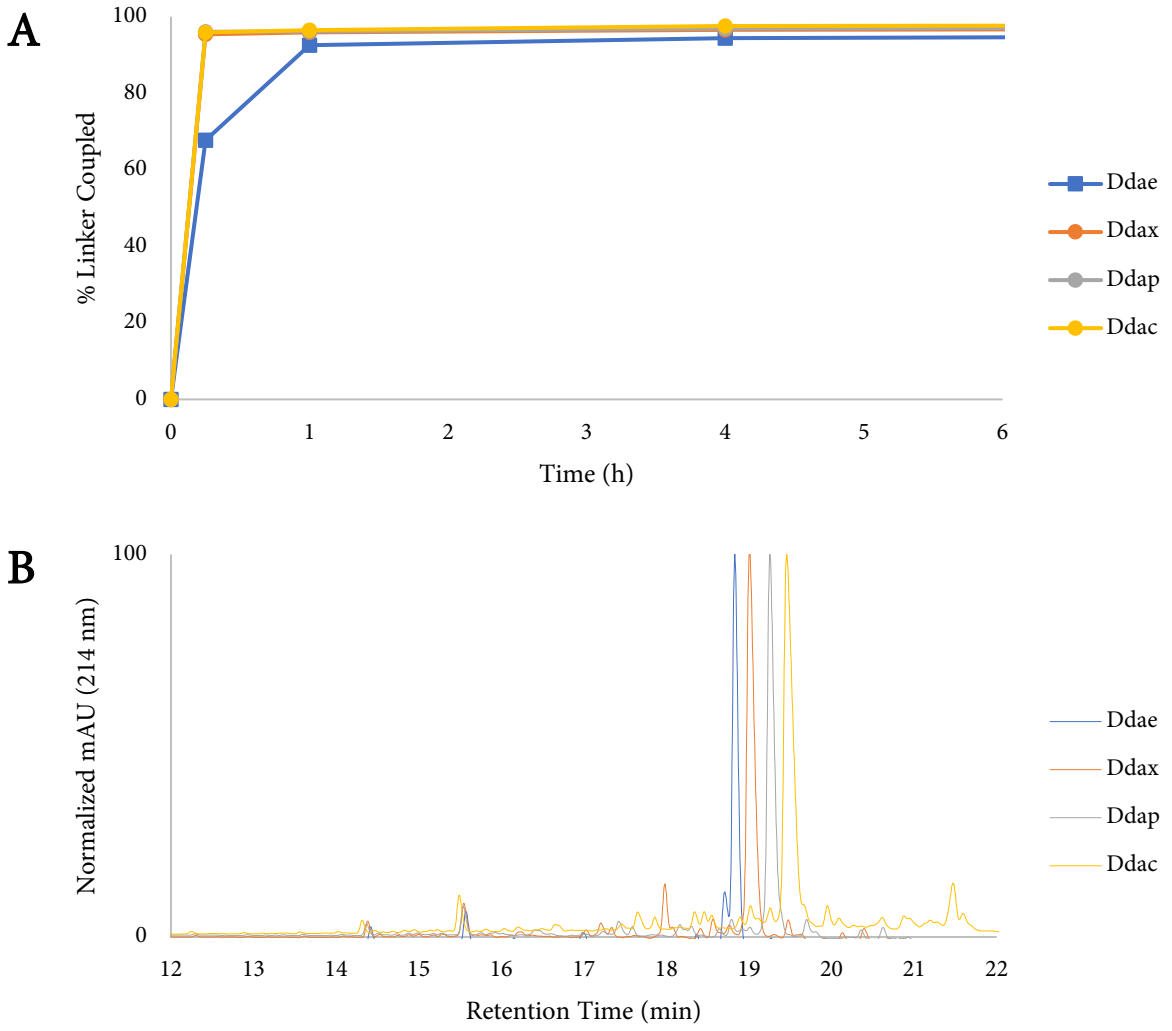

Figure S19: (A) Comparison of linker coupling with C20 peptide on Tentagel R Ram Resin and 1 mL NMP, 200 mM linker (37°C) at 0.25, 1, 4, and 24 h (24 h timepoint not shown). Reactions were quenched by removing ~3 μmol resin at each timepoint and washing thoroughly with DMF and DCM before TFA cleavage followed by ether precipitation. **(B)** HPLC analysis of crude C20 peptides with indicated linkers after 24 h using **Analytical Method A**

After coupling of the linker, addition of the Lys_6 solubilizing tag was accomplished using standard SPPS procedures.

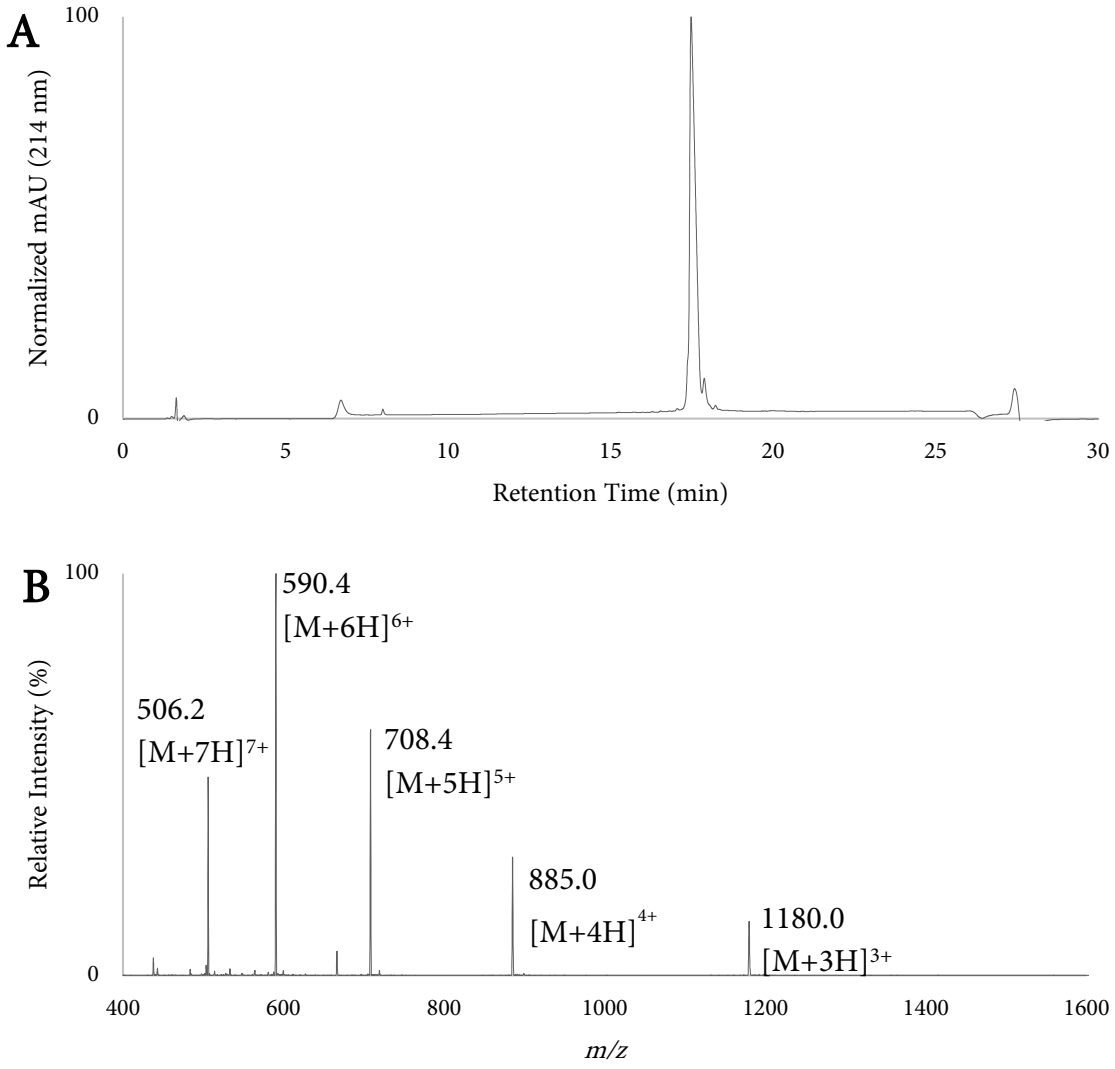

Figure S20: (A) C20(Lys$_6$-Ddae) (Ac-DWTKNITDK(**KKKKKK-Ddae**)IDQIIHDFVDK-NH_2; 48.3 mg, 14 μmol) was HPLC purified using **Purification Method A** to give 16.4 mg pure peptide (15% yield) before analysis with **Analytical Method A** (t_R= 17.5 min). **(B)** Mass spectrum of pure C20(Lys$_6$-Ddae) was determined using **LC-MS Method A.** Calculated mass = 3536.20 Da, Observed mass = 3536.3 Da

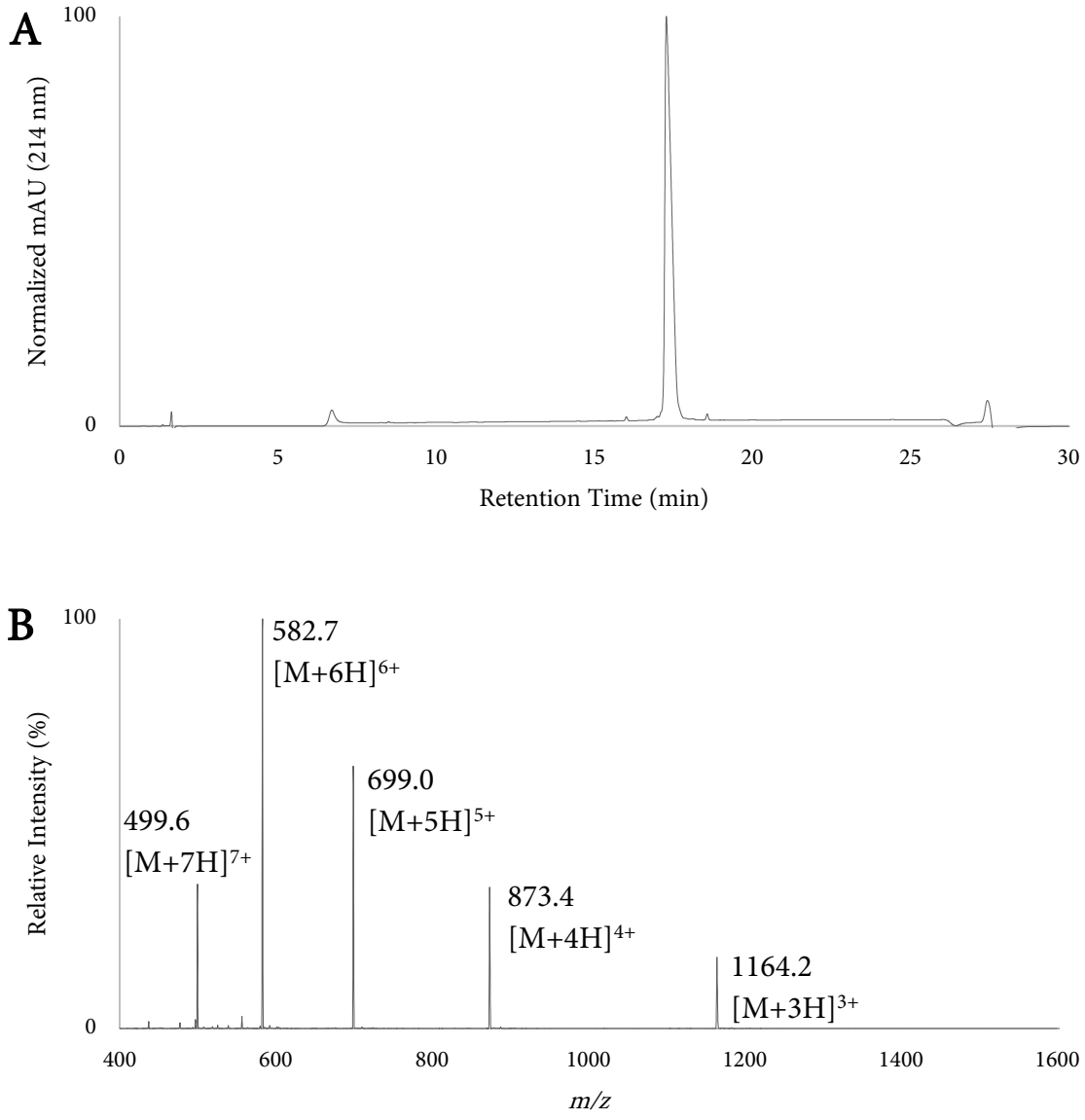

Figure S21: (A) C20(Lys$_6$-Ddax) (Ac-DWTKNITDK(**KKKKKK-Ddax**)IDQIIHDFVDK-NH2; 64.5 mg, 18 μmol) was HPLC purified using **Purification Method A** to give 20.2 mg pure peptide (19% yield) before analysis using **Analytical Method A** (t_R= 17.3 min). **(B)** Mass spectrum of pure C20(Lys$_6$-Ddax) was determined with **LC-MS Method A**. Calculated mass = 3490.10 Da, Observed mass = 3490.3 Da

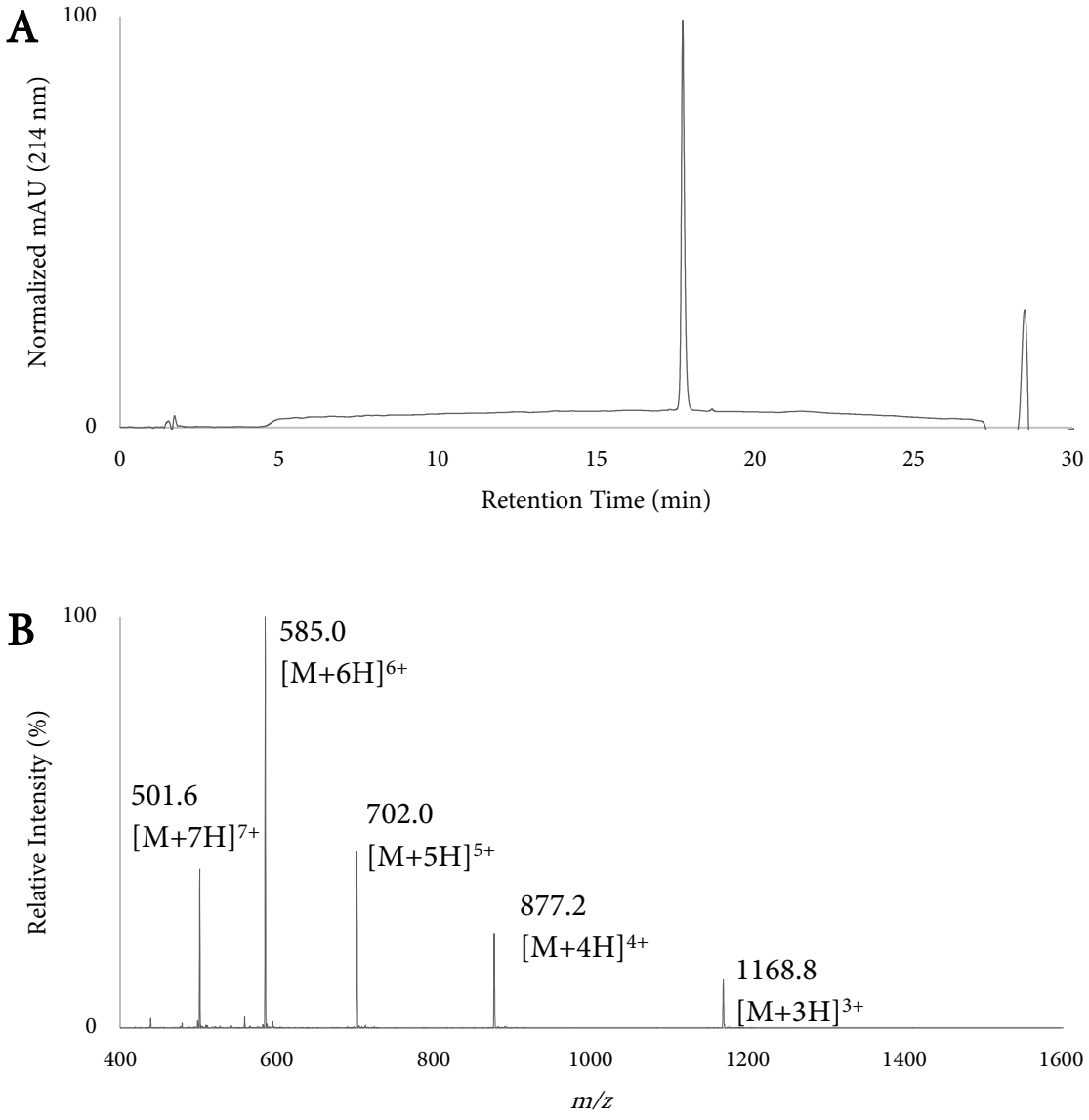

Figure S22: (A) C20(Lys$_6$-Ddap) (Ac-DWTKNITDK(**KKKKKK-Ddap**)IDQIIHDFVDK-NH$_2$; 70.3 mg, 20 μmol) was HPLC purified using **Purification Method A** to give 19.8 mg pure peptide (19% yield) before analysis with **Analytical Method A** (t_R= 17.7 min)**. (B)** Mass spectrum of pure C20(Lys$_6$-Ddap) was determined using **LC-MS Method A**. Calculated mass = 3504.10 Da, Observed mass = 3504.3 Da

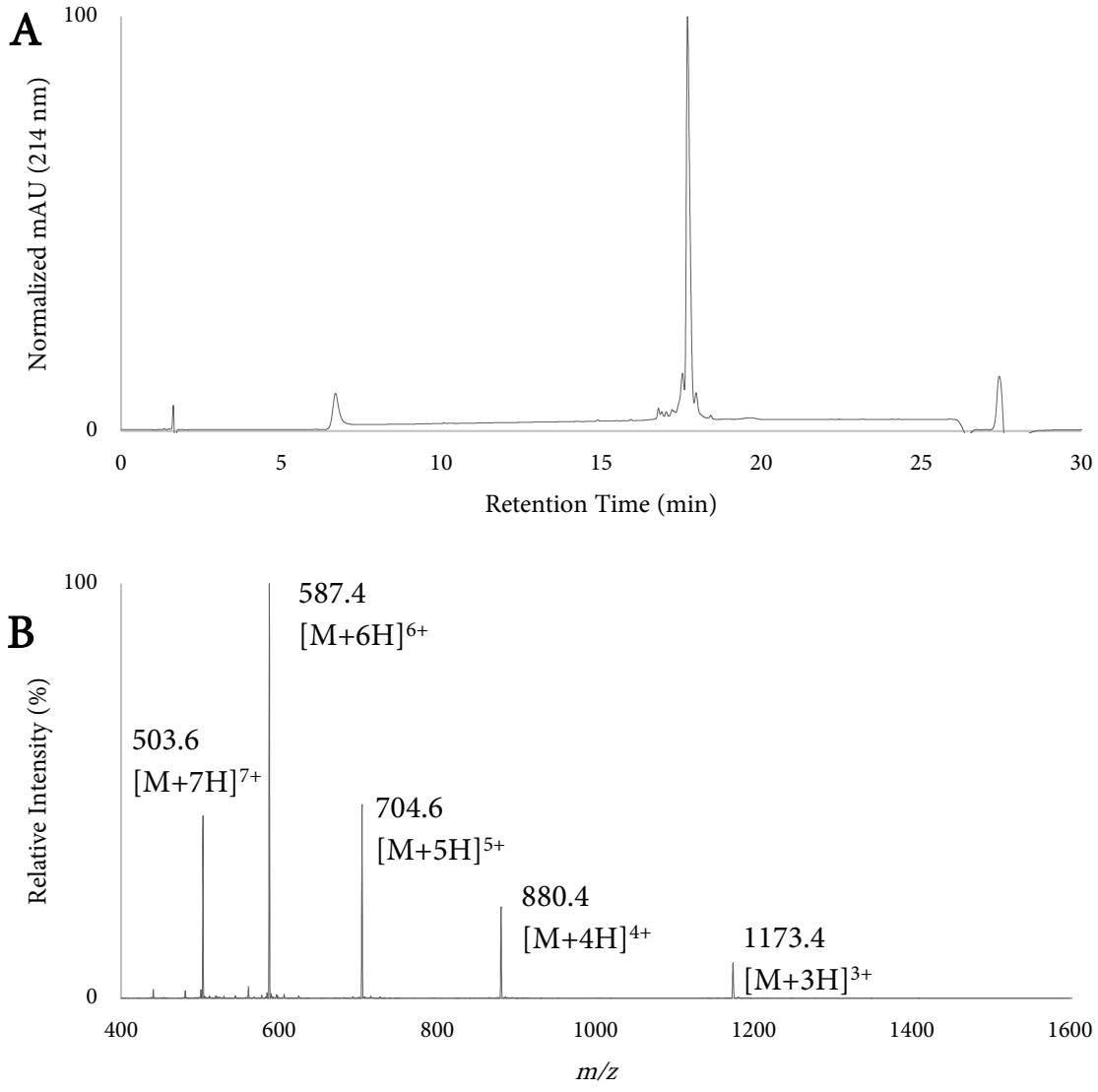

Figure S23: (A) C20(Lys$_6$-Ddac) (Ac-DWTKNITDK(**KKKKKK-Ddac**)IDQIIHDFVDK-NH_2; 67.4 mg, 19 μmol) was HPLC purified using **Purification Method A** to give 25.4 mg pure peptide (24% yield) before analysis with **Analytical Method A** (t_R= 17.7 min)**. (B)** Mass spectrum of pure C20(Lys$_6$-Ddac) was determined using **LC-MS Method A**. Calculated mass = 3518.10 Da, Observed mass = 3518.3 Da

Section XI: Initial Linker Cleavage Conditions

For the initial comparison of cleavage kinetics for each linkers, preparation of cleavage buffer was performed as follows:

- 8 mL 6 M GuHCl, 100 mM phosphate, pH 3
- Add 750 µL of 12 M HCl and vortex
- Add 327 µL of anhydrous hydrazine and vortex
- pH adjust using 12 M HCl until the solution is pH 7.5
- Adjust to a final volume of 10 mL with 6 M GuHCl, 100 mM phosphate, pH 7.5
- Filter solution using 0.2 µm nylon syringe filter

Lyophilized C20 peptides with helping hand were dissolved directly into this cleavage buffer to a concentration of ~0.5 mM and adjusted to a final pH of 7.5. All timepoints were quenched through the addition of 80 µL 10% AcOH to 20 µL sample before analysis by HPLC.

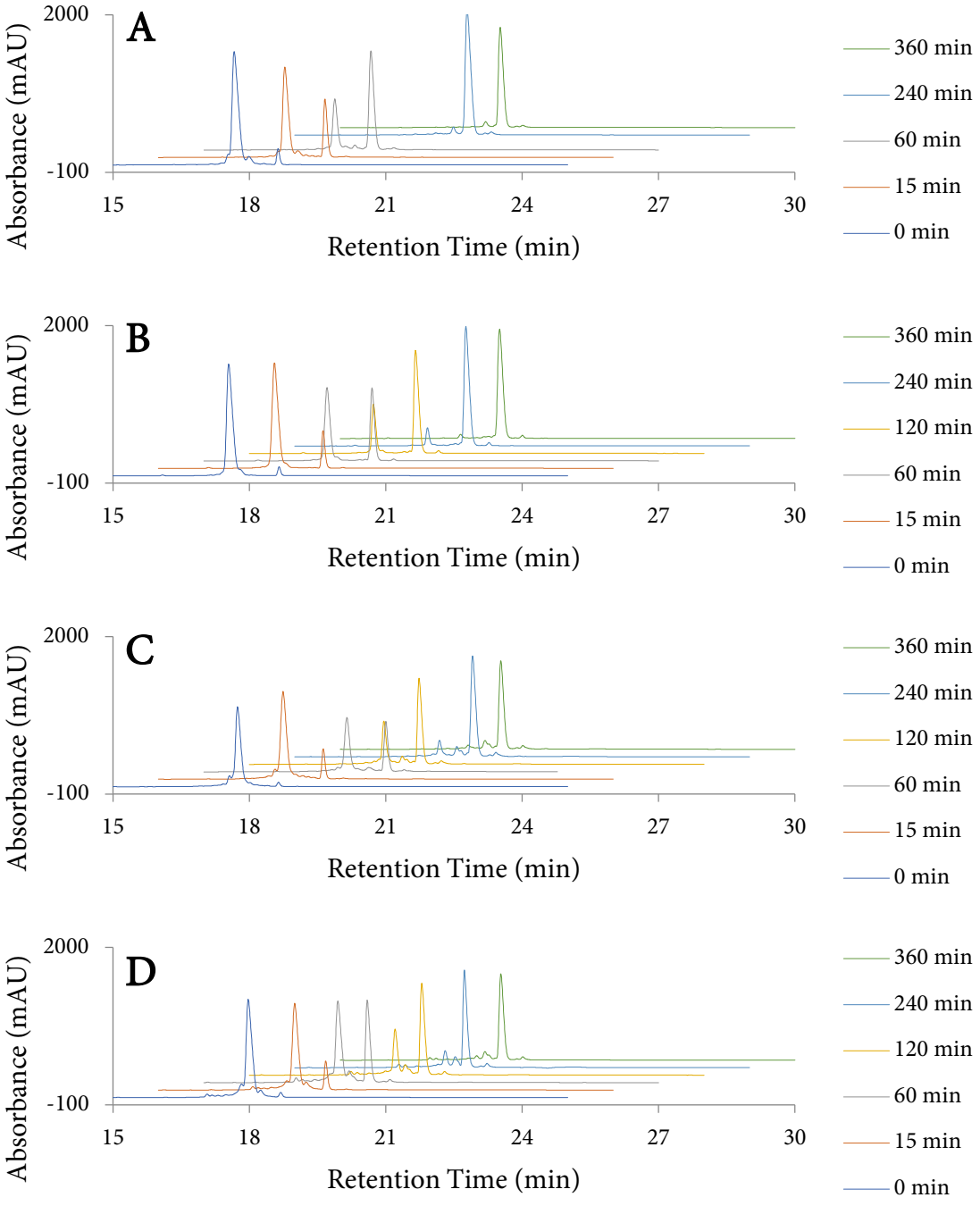

Figure S24: (A) Cleavage of C20(Lys$_6$-Ddae) monitored with **Analytical Method A.** Note: The 120 min timepoint is omitted for clarity due to a t_R shift. **(B)** Cleavage of C20(Lys$_6$-Ddax) monitored with **Analytical Method A. (C)** Cleavage of C20(Lys$_6$-Ddap) monitored with **Analytical Method A. (D)** Cleavage of C20(Lys$_6$-Ddac) monitored with **Analytical Method A.**

Section XII: Determination of Rate Constants

HPLC peak areas for all samples were integrated using Agilent Chemstation. To calculate "% Linker Cleaved," the equation detailed in Figure S25 was used**.** Briefly, the integrated peak area for peptide without linker was multiplied with a correction factor (CF = 27/21) and divided by the sum of the peptide with and without helping hand before normalization to 100. The CF was determined based on the number of amide bonds with the helping hand (27) divided by the number of amide bonds without helping hand (21). This CF was utilized to account for the loss of A_{214} from the linker with the Lys_6 tag. The linker and Lys_6 tag peak area could be integrated on several of the runs but not all due to interference from buffer components. In the cases where the linker with Lys_6 tag peak area was capable of integration, we utilized that data to validate the CF and found both values to be within 2% of each other (data not shown). Data generated following the equation in Figure S25 was imported into GraphPad Prism 8.1.2. The non-linear regression "one-phase association" (with plateau constrained to 100 and $k > 0$) was used to determine the pseudo-first-order rate constant k (s^{-1}).

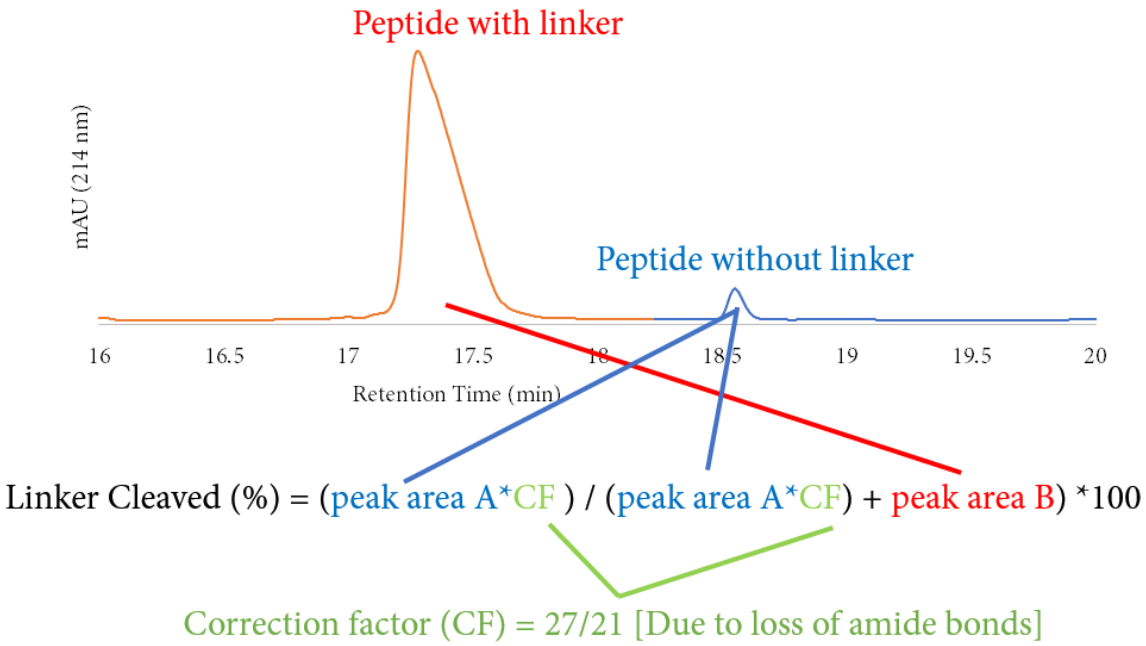

Figure S25: Equation used for calculating "Linker Cleaved (%)." To calculate "Uncleaved Linker (%)," peak area A*CF and peak area B are switched within the equation.

Section XIII: Comparison of Ddae and Ddap Stability

To determine stability of the Ddap linker in commonly encountered reaction conditions, C20(Lys_6-Ddap) was dissolved to 0.5 mM in the following buffers and monitored by HPLC over 48 h:

Buffer A: 0.1%TFA in 50% ACN (HPLC Buffer)

Buffer B: 6 M GnHCl, 100 mM $NaPO_4$, pH 3

Buffer C: 6 M GnHCl, 200 mM $NaPO_4$, pH 7

Buffer D: 6 M GnHCl, 5% AcOH

Buffer E: 6 M GnHCl, 100 mM $NaPO_4$, 200 mM $MeONH_2$, pH 3 (Thz cleavage conditions)

Buffer F: 6 M GnHCl, 200 mM $NaPO_4$, 200 mM MPAA, 50 mM TCEP, pH 7 (NCL conditions)

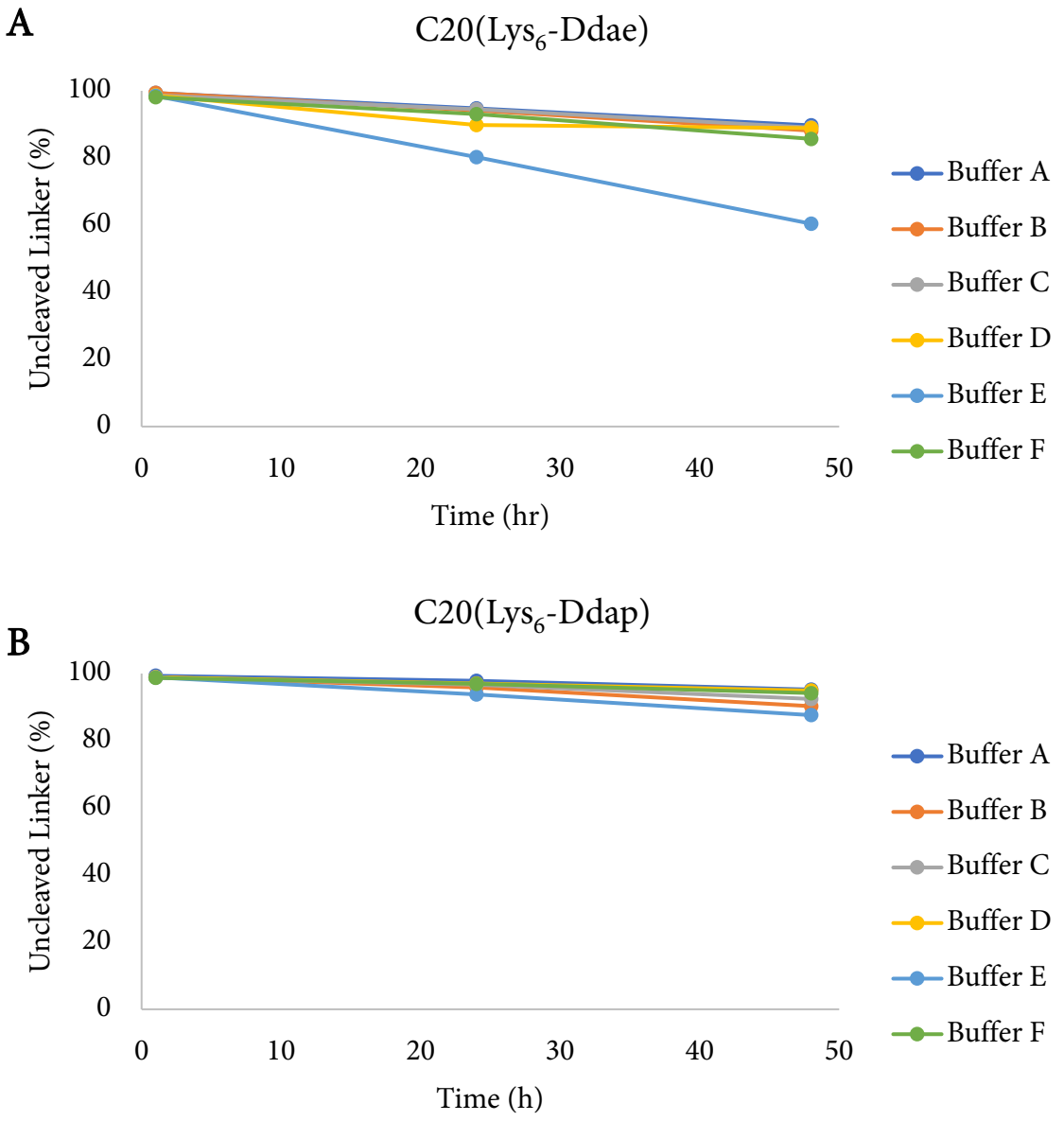

Figure S26: A) Stability of C20(Lys$_6$-Ddae) in various buffers commonly used in peptide synthesis. Stability data for Ddae were collected from previously published results[2] and analyzed following the equation in Figure S25. **B)** Stability of C20(Lys$_6$-Ddap) in various buffers commonly used in peptide synthesis**.** Data were collected in duplicate with independent reactions using **Analytical Method A**.

Section XIV: Cleavage rates and side-reactions with hydroxylamine or hydrazine

For the rate cleavage comparison between hydrazine and hydroxylamine, we adjusted our cleavage protocol to utilize the safer HCl salts of each nucleophile (hydrazine monohydrochloride and hydroxylamine hydrochloride). To generate "cleavage buffer" containing 1 M hydrazine or 1 M hydroxylamine the following protocol was followed: 205.5 mg (3 mmol) hydrazine monohydrochloride and 208.5 mg (3 mmol) hydroxylamine monohydrochloride were weighed into 5 mL volume Eppendorf tubes. 2 mL ligation buffer (6 M GnHCl, 200 mM $NaPO_4$, pH 7) was added to each tube and pH adjusted to 6.75 for hydroxylamine or 7.5 for hydrazine using 10 M NaOH before being diluted to 3 mL with pH 6.75 or 7.5 ligation buffer. These solutions were filtered with a 0.2 μm nylon syringe filter before use. C20(Lys_6-Ddap) peptide was dissolved in both solutions to a concentration of 0.5 mM before analysis by HPLC. Reactions were quenched at all timepoints by adding 80 μL 5% AcOH to 20 μL reaction. Data were collected in duplicate with independent reactions using **Analytical Method A**.

	1 M Hydroxylamine pH 6.75	1 M Hydrazine pH 7.5
k (10^{-3} s^{-1})	224.8	11.6
95% CI	137.1-365.8	9.7-13.9
R square	0.9415	0.9833
n	2	2

Table S3: Curve fit using non-linear regression "one-phase association" in Prism 8.1.2.

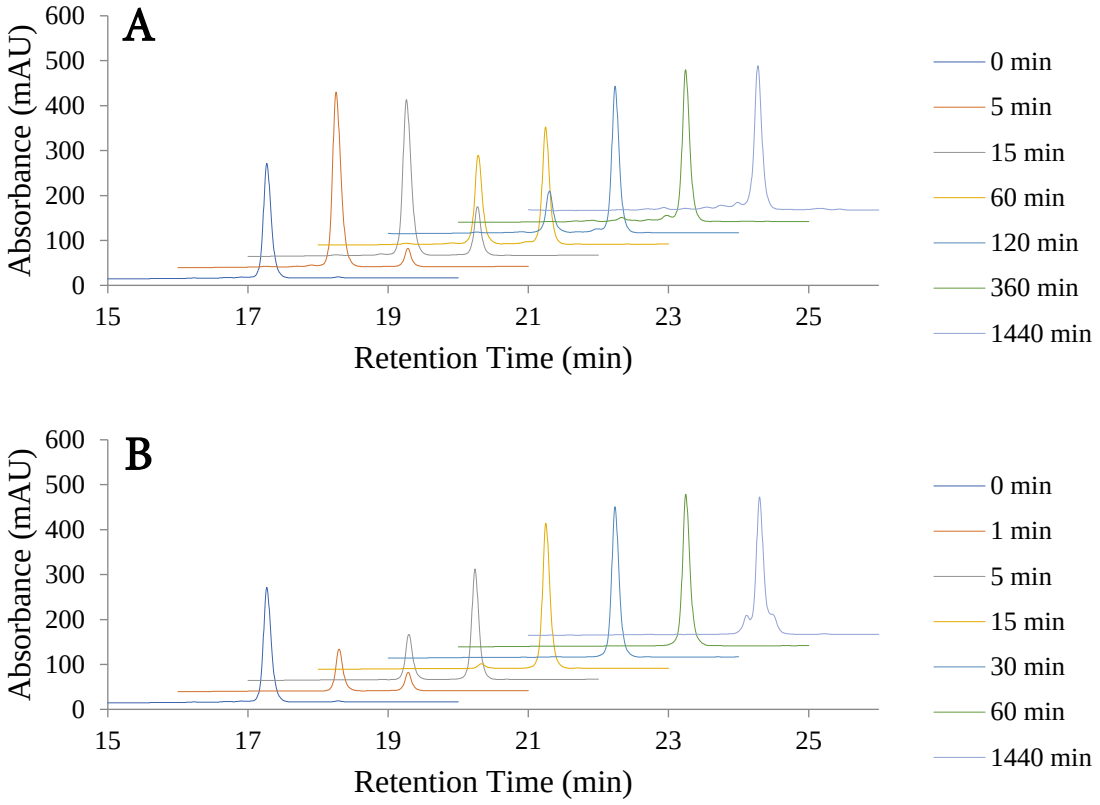

Figure S27: (A) Representative HPLC traces of C20(Lys_6-Ddap) cleavage using 1 M hydrazine in cleavage buffer, pH 7.5 (**Analytical Method A). (B)** Representative HPLC traces of C20(Lys_6-Ddap) cleavage using 1 M hydroxylamine in cleavage buffer, pH 6.75 (**Analytical Method A)**.

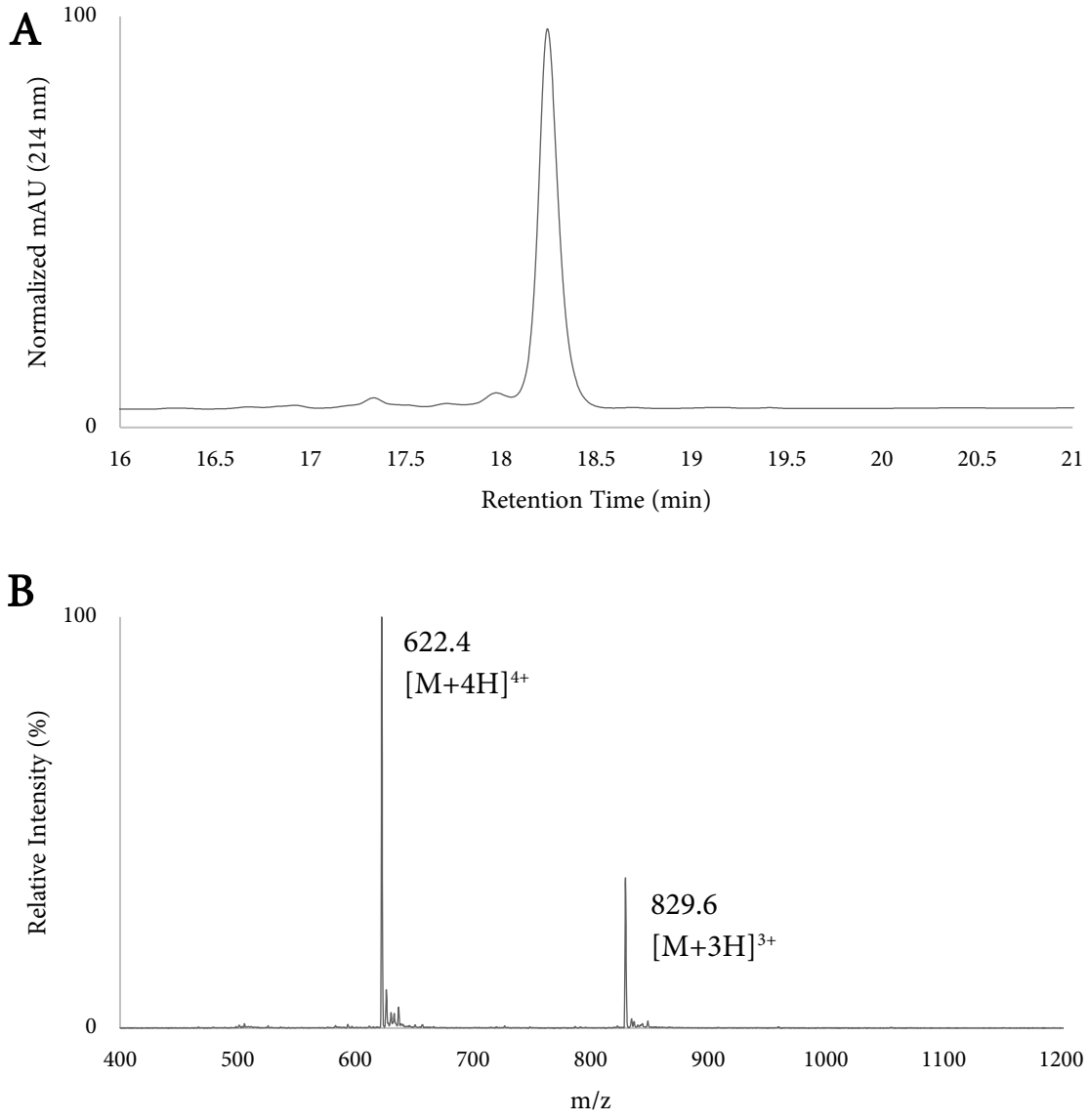

Figure S28: (A) HPLC chromatogram of C20(Lys_6-Ddap) after incubation with 1 M hydrazine in cleavage buffer (pH 7.5) for 360 min (**Analytical Method A). (B)** Mass spectrum of cleaved C20 after incubation with 1 M hydrazine for 360 min (**LC-MS Method D**). Calculated mass = 2485.70 Da, Observed mass = 2485.7 Da.

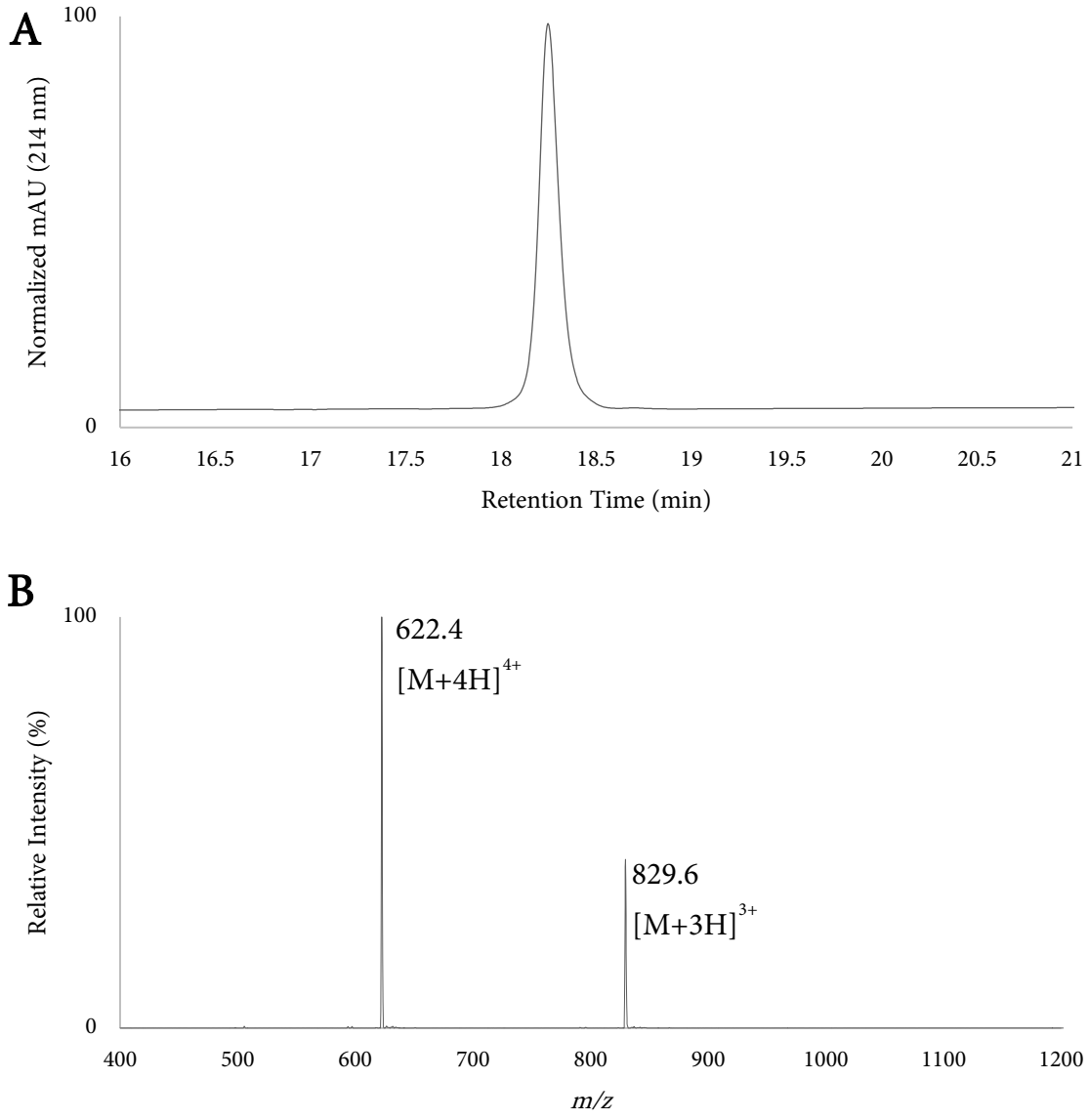

Figure S29: (A) HPLC chromatogram of C20(Lys_6-Ddap) after incubation with 1 M hydroxylamine in cleavage buffer (pH 6.75) for 60 min (**Analytical Method A). (B)** Mass spectrum of cleaved C20 after incubation with 1 M hydroxylamine for 60 min (**LC-MS Method D**). Calculated mass = 2485.70 Da, Observed mass = 2485.7 Da.

The 1 M hydrazine pH 7.5 and 1 M hydroxylamine pH 6.75 reactions were further incubated for 1440 min to identify any side-products that can occur from extended treatment.

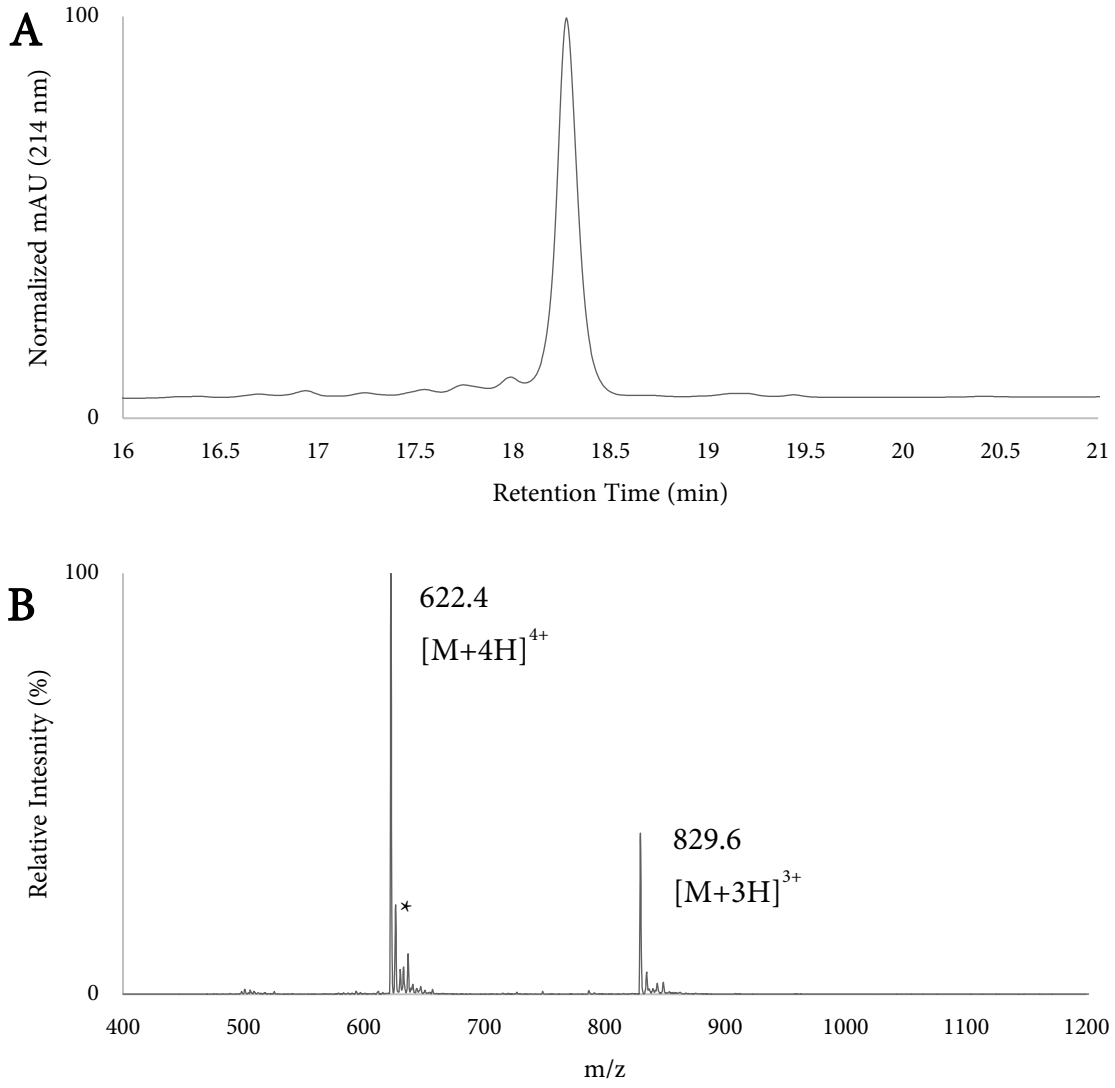

Figure S30: (A) HPLC chromatogram of C20(Lys$_6$-Ddap) after incubation with 1 M hydrazine in cleavage buffer (pH 7.5) for 1440 min (**Analytical Method A). (B)** Mass spectrum of cleaved C20 after incubation with 1 M hydrazine for 1440 min (**LC-MS Method D**). Calculated mass = 2485.70 Da, Observed mass = 2485.7 Da. * indicates side products with the following masses: 2501.2 Da, 2527.6 Da, and 2542.7 Da.

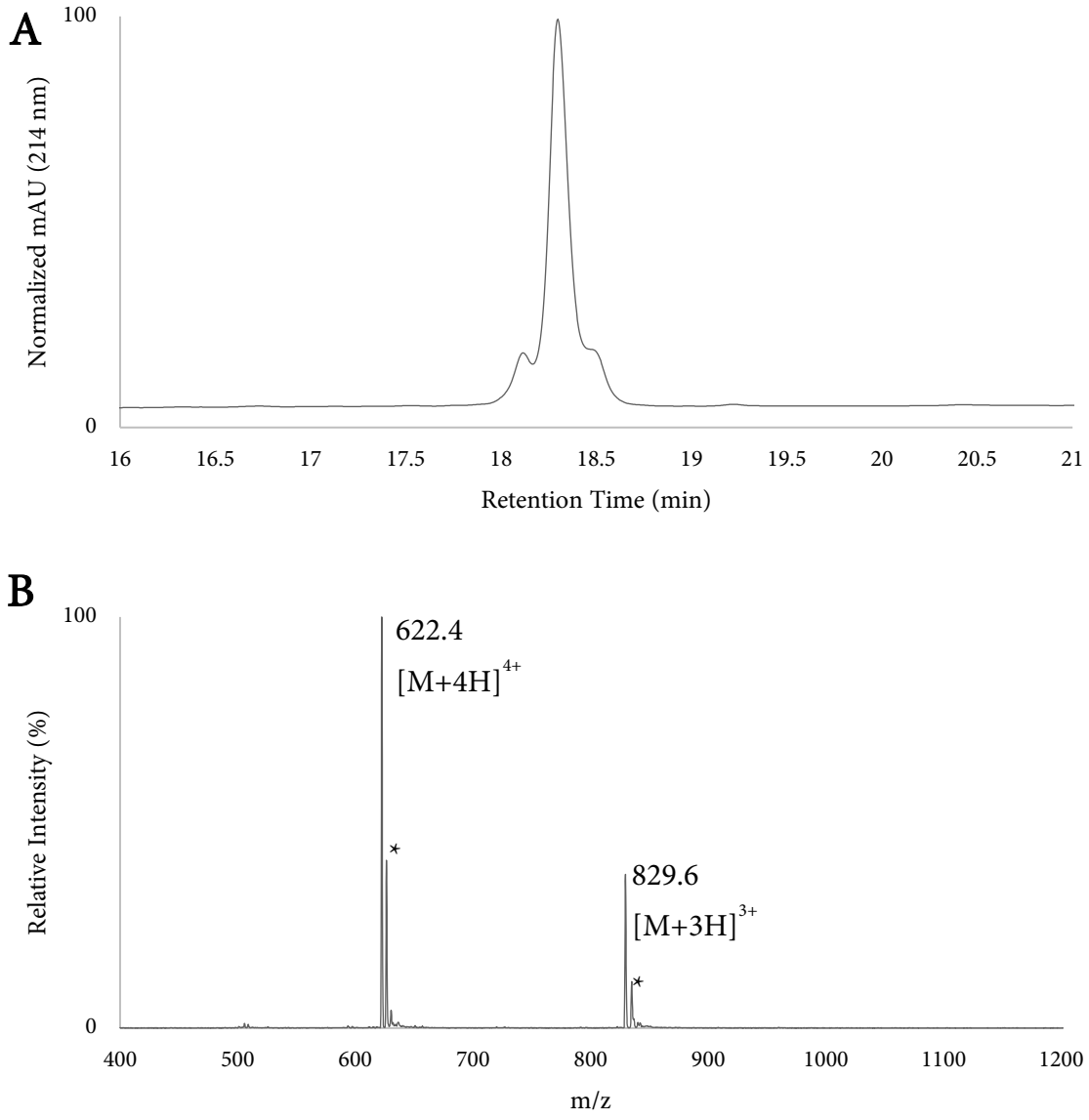

Figure S31: (A) HPLC chromatogram of C20(Lys_6-Ddap) after incubation with 1 M hydroxylamine in cleavage buffer (pH 6.75) for 1440 min (**Analytical Method A). (B)** Mass spectrum of cleaved C20 after incubation with 1 M hydroxylamine for 1440 min (**LC-MS Method D**). Calculated mass = 2485.70 Da, Observed mass = 2485.7 Da. * indicates side products with the following masses: 2501.7 Da, 2517.3 Da, and 2540.2 Da.

Section XV: Determination of Ddap A_{280} extinction coefficient

For peptides Fl-C20 and Fl-C20(Ddap), 5,(6)-carboxyfluorescein was coupled to N-termini using standard coupling conditions. Fl-C20 was synthesized at 10 μmol scale while Fl-C20(N_3-Ddap) was synthesized at 20 μmol scale.

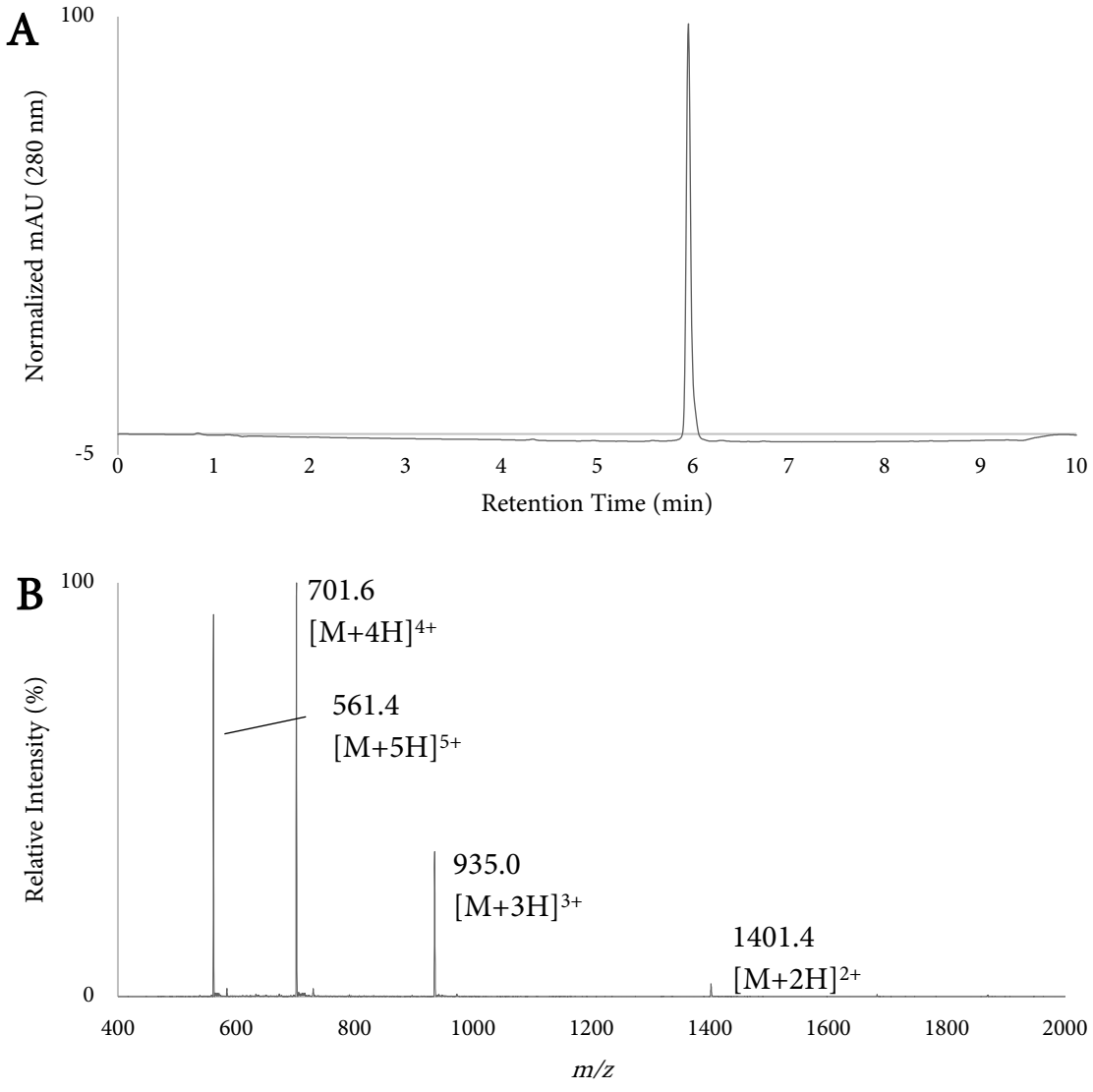

Figure S32: (A) Fl-C20 (Fl-DWTKNITDKIDQIIHDFVDK-NH_2; 13 mg, 5 μmol) was HPLC purified using **Purification Method B** to give 4.5 mg pure peptide (16% yield) before analysis

with **LC-MS Method A** (t_R= 5.9 min). **(B)** Mass spectrum of pure Fl-C20 was determined using **LC-MS Method A.** Calculated mass = 2802.02 Da, Observed mass = 2802.1 Da

With Fl-C20(N_3-Ddap), the linker was capped with the compound 6-azidohexanoic acid using standard SPPS coupling conditions.

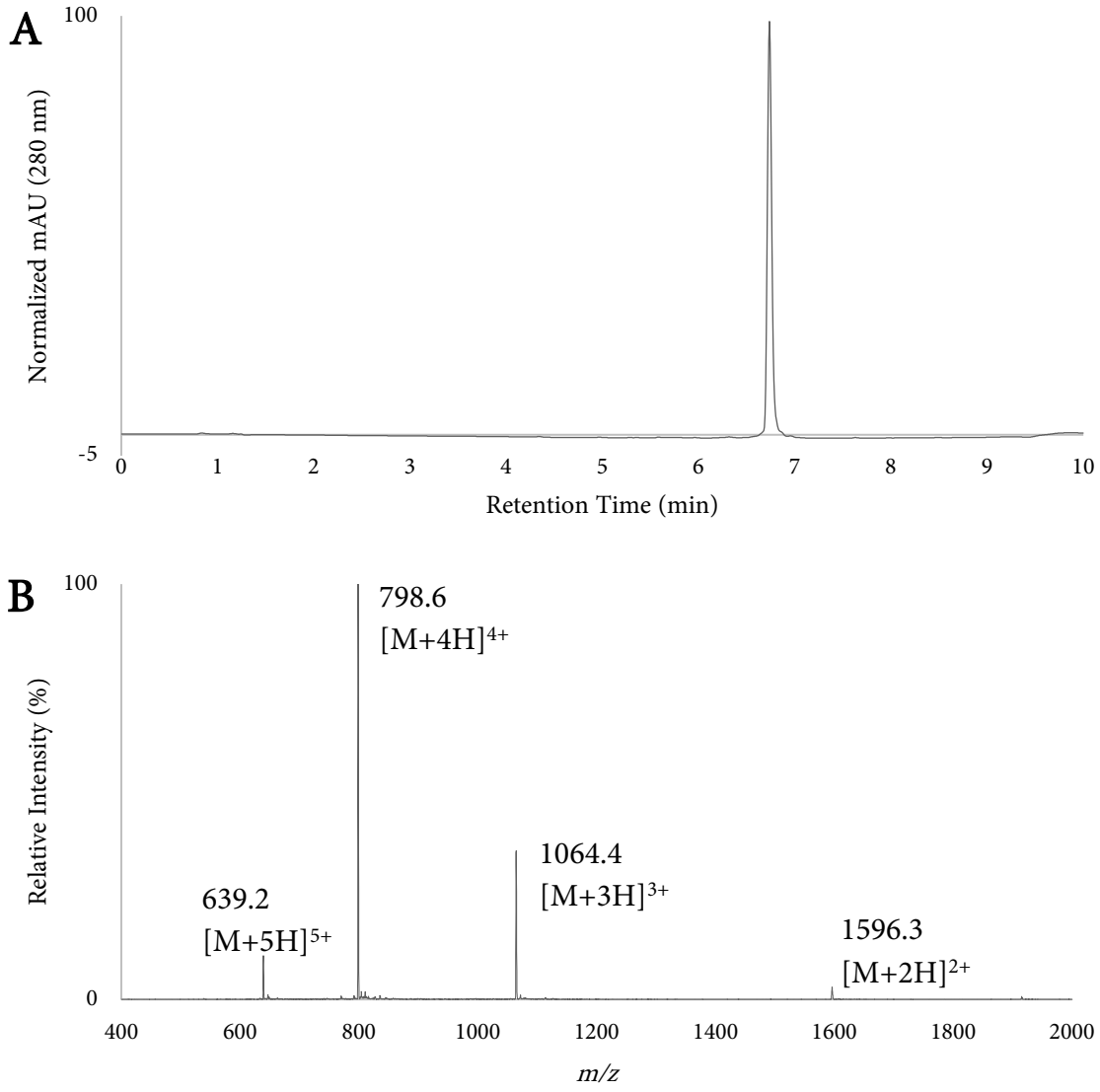

Figure S33: (A) Fl-C20(N_3-Ddap) (Fl-DWTKNITDK(**6-azido-Ddap**)IDQIIHDFVDK-NH_2; 34 mg, 11 µmol) was HPLC purified using **Purification Method B** to give 6.3 mg pure peptide (10%

yield) before analysis with **LC-MS Method A** (t_R= 6.7 min). **(B)** Mass spectrum of pure Fl-C20(N_3-Ddap) was determined using **LC-MS Method A.** Calculated mass = 3190.54 Da, Observed mass = 3190.7 Da

Fl-C20 and Fl-C20(N_3-Ddap) were dissolved in ligation buffer (6 M GnHCl, 200 mM $NaPO_4$, pH 8) before measuring the A_{495} using a NanoDrop 2000 (ThermoFisher). Using the A_{495} and ε of fluorescein at 495 nm (77,000 $M^{-1} \cdot cm^{-1}$),[5] the concentration of both peptides was adjusted to be equal (0.26 mM). The A_{280} of both peptides was then compared and found to have a ΔA_{280} of 3.8. Applying Beer-Lambert law ($A = \varepsilon \cdot L \cdot c$), the ε of Ddap was determined to be ~14,600 $M^{-1} \cdot cm^{-1}$.

Section XVI: Synthesis and Characterization of StxB peptides

StxB peptides were synthesized following standard synthesis procedures described previously with several exceptions noted below. Pseudoproline dipeptides were utilized at indicated positions (in bold) in Table S4 to improve the synthetic quality of StxB-N. Heated couplings (50 °C, in red) and double couplings (underline) were utilized at indicated positions in Table S4. Lys(Dde)-OH was incorporated at the indicated position (marked with X) to allow linker addition. Boc-Thr(tBu)-OH was utilized as the N-terminal amino acid on StxB-N.

Peptide	Sequence
StxB-N	TPDCVTGKVEYTKYNDD**DT**FTVKVGDXEL**FT**NRW NLQSLLL**LS**AQITGMT**VT**IKTNA—$NHNH_2$
StxB-N(HH)	TPDCVTGKVEYTKYNDD**DT**FTVKVGDK(KKKKKK-Ddap) EL**FT**NRWNLQSLLL**LS**AQITGMT**VT**IKTNA—$NHNH_2$
StxB-C	CHNGGGFSEVIFR—OH

Table S4: Sequences of all StxB peptides used in this study. The StxB-N sequence also details non-standard procedures during synthesis to overcome difficult amino acid couplings and deprotections. Red indicates amino acid couplings performed at 50 °C, underline indicates double couplings, bold indicates the use of pseudoproline dipeptides, and X indicates the position of Lys(Dde)

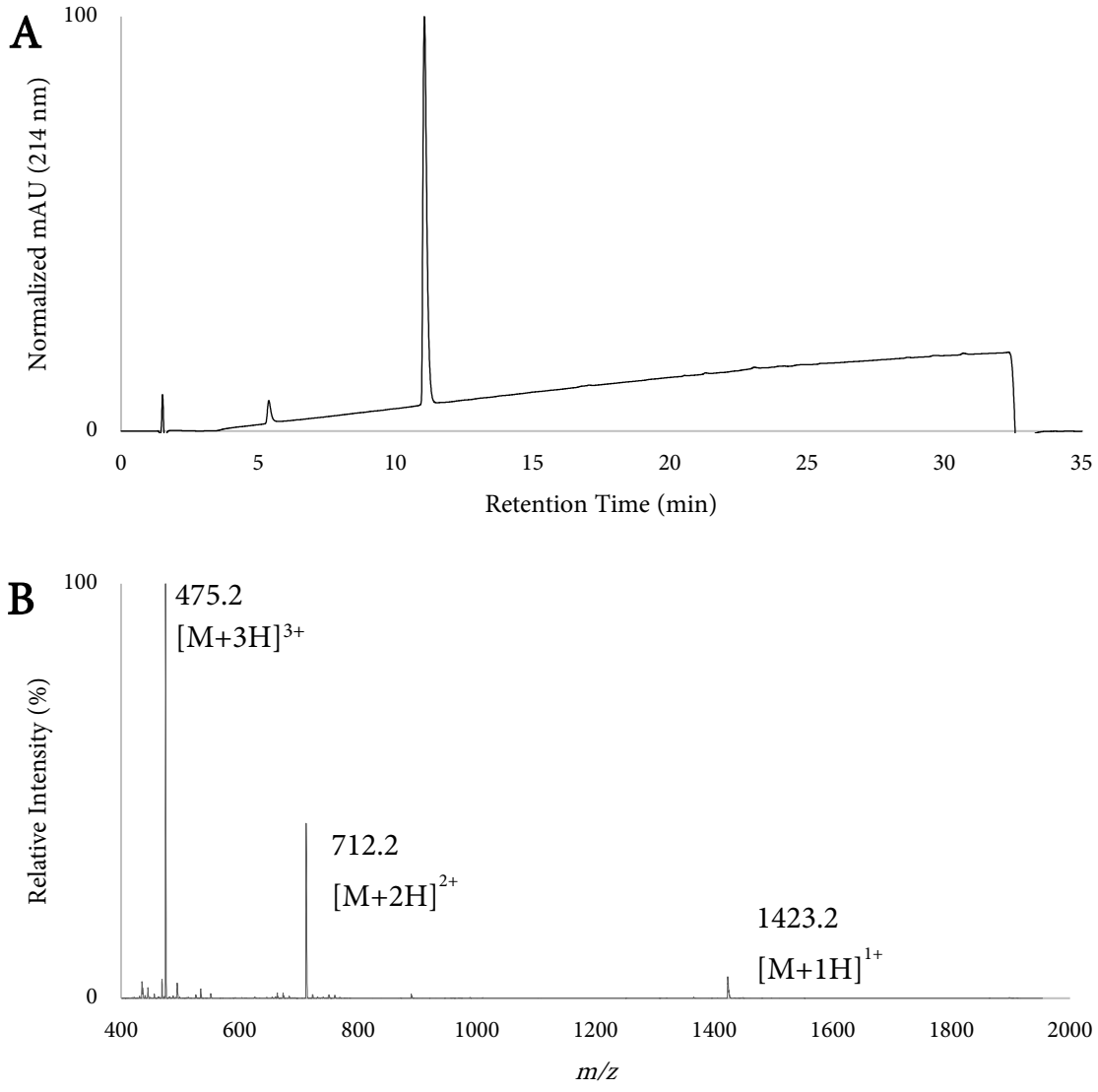

Figure S34: (A) StxB-C (CHNGGGFSEVIFR-OH; 29.7 mg, 21 μmol) was HPLC purified using **Purification Method C** to give 21.0 mg pure peptide (49% yield) before analysis using **Analytical Method B** (t_R= 11.1 min). **(B)** Mass spectrum of pure StxB-C was determined using **LC-MS Method A.** Calculated mass = 1422.56 Da, Observed mass = 1422.2 Da

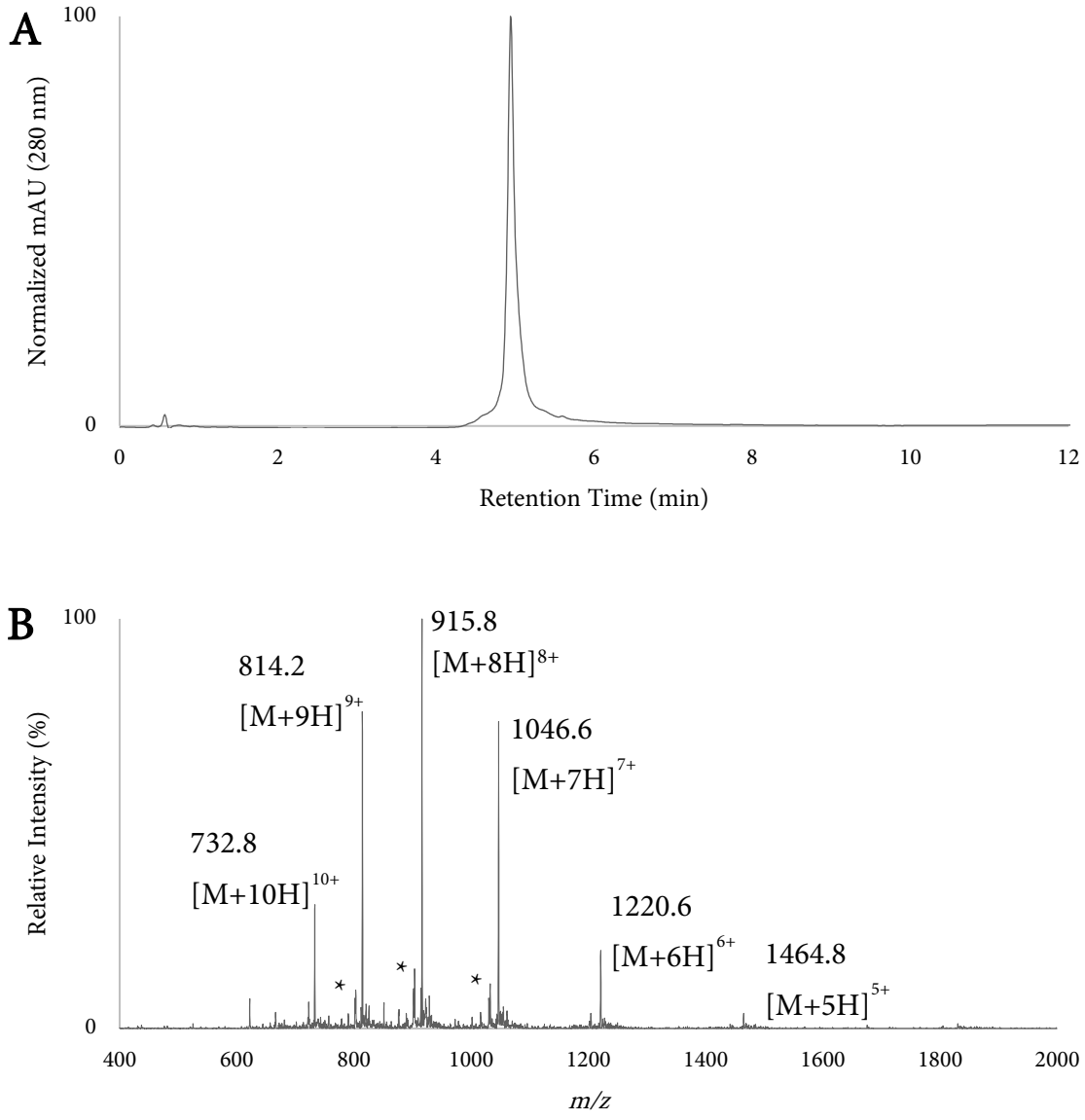

Figure S35: (A) StxB-N(HH) (TPDCVTGKVEYTKYNDDDTFTVKVGDK(**KKKKKK-Ddap**)ELFTNRWNLQSLLLSAQITGMTVTIKTNA—$NHNH_2$; 136.0 mg, 18.6 μmol) was HPLC purified using **Purification Method D** to yield 20.0 mg pure peptide (9% yield) before analysis using **LC-MS Method B** (t_R= 4.9 min)**. (B)** Mass spectrum of pure StxB-N(HH) was determined using **LC-MS Method B.** Calculated mass = 7319.45 Da, Observed mass = 7319.0 Da. * indicates Val deletion (-99 Da).

Section XVII: Native Chemical Ligation of StxB-N(HH) and StxB-C

Native chemical ligation was performed according to standard methods[6,7] using MPAA as thiol catalyst and TCEP as reducing agent. The hydrazide method was used to generate C-terminal thioesters.[1,8,9] StxB-N(HH) (5 mg, 0.7 μmol) was dissolved in 675 μL "activation buffer" (6 M GuHCl, 100 mM $NaPO_4$, pH 3) and activated (conversion of hydrazide to acyl azide) with freshly prepared sodium nitrite (20 mM final concentration $NaNO_2$) for 20 min at -20 ° C. During activation, a solution containing 200 mM MPAA in "ligation buffer" (6 M GuHCl, 200 mM phosphate, pH 7) was prepared. 3 equiv. of StxB-C (3 mg, 2.1 μmol) were dissolved in 675 μL of this solution. Both solutions were combined, and the final pH was adjusted to 7.0-7.2 to initiate the ligation after activation of the hydrazide. After 15 min, 150 μL 0.5 M TCEP in pH 7 ligation buffer was added to the reaction to a final concentration of 50 mM.

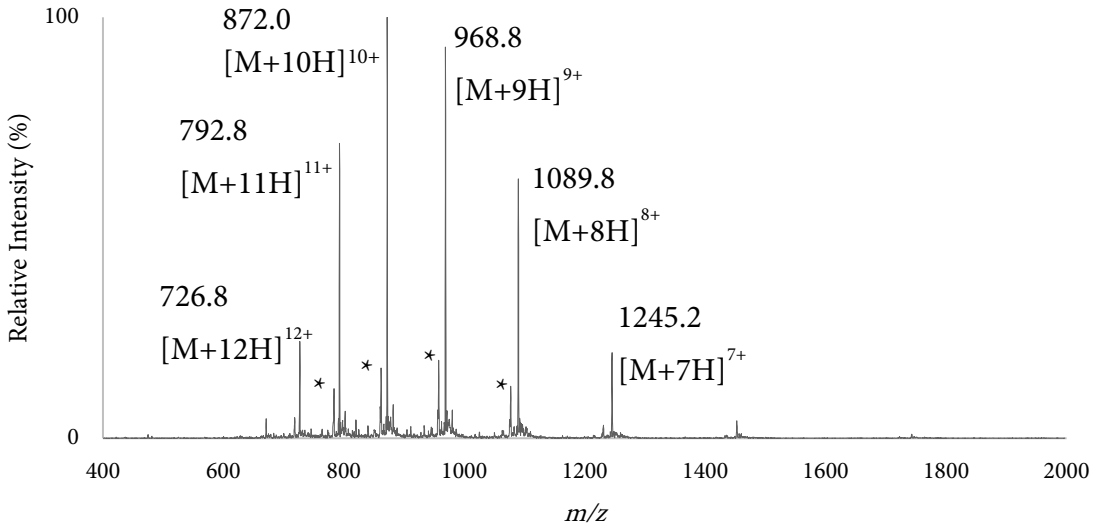

Figure S36: Mass spectrum of StxB-(HH) was determined using **LC-MS Method B.** Calculated mass = 8709.01 Da, Observed mass = 8709.8 Da. * indicates Val deletion (-99 Da).

Linker cleavage after the NCL reaction was accomplished through a one-pot reaction with the addition of 1.5 mL 2 M hydroxylamine in cleavage buffer (6 M GnHCl, 100 mM PO_4, pH 6.75). After completion of helping hand cleavage, 3 mL of 10% AcOH was added. The solution was centrifuged at 5000 g to remove any aggregates. The supernatant was then filtered with 0.2 μm nylon filter before HPLC purification.

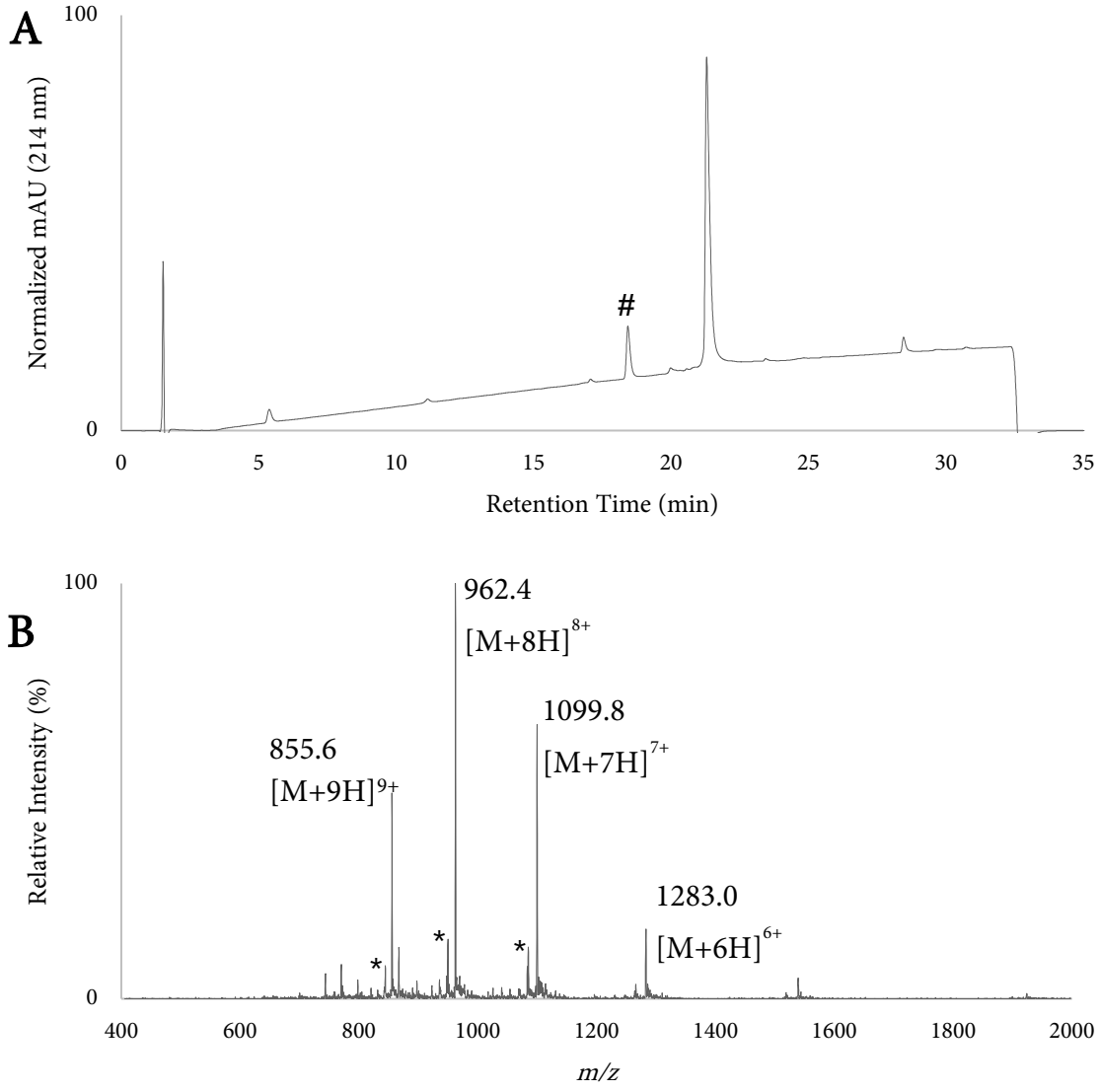

Figure S37: (A) StxB (TPDCVTGKVEYTKYNDDDTFTVKVGDKELFTNRWNLQ

SLLLSAQITGMTVTIKTNACHNGGGFSEVIFR—OH) was HPLC purified using **Purification Method E** before analysis with **Analytical Method B** (t_R= 21.3 min)**. #** indicates oxidized-MPAA contaminant that coeluted with full length StxB during final HPLC purification**. (B)** Mass spectrum of StxB was determined using **LC-MS Method B.** Calculated mass = 7690.62 Da, Observed mass = 7691.3 Da. * indicates Val deletion (-99 Da).

Section XVIII: Oxidative Dialysis of StxB

Lyophilized full-length StxB was dissolved in 0.8 mL oxidation buffer (7 M GnHCl, pH 8.0, 2% DMSO) and adjusted to a concentration of 40 μM. This solution was incubated for 24 h at 37 °C to allow for intramolecular disulfide formation (confirmed by MS analysis) and then transferred to 0.5 mL Slide-A-Lyzer mini-dialysis cassettes (3,500 MWCO) for step-wise dialysis into the following solutions at 4°C:

1) 0.6 L, 3 M GnHCl, 50 mM $NaPO_4$, pH 8.0, 5 mM EDTA for 12 h

2) 0.6 L, 1 M GnHCl, 50 mM $NaPO_4$, pH 8.0, 1 mM EDTA for 24 h

3) 1 L, Phosphate Buffered Saline (50 mM $NaPO_4$, 150 mM NaCl, pH 7.4) for 4 h

4) 1 L, Phosphate Buffered Saline (50 mM $NaPO_4$, 150 mM NaCl, pH 7.4) for 4 h

5) 1 L, Phosphate Buffered Saline (50 mM $NaPO_4$, 150 mM NaCl, pH 7.4) for 12 h

Dialyzed material was then clarified by centrifugation at 21,000 g (4 °C) for 20 min before further biochemical analysis. Folded synthetic Stxb was compared to recombinant StxB purchased from

Sigma (catalogue # SML0562). 0.5 mg lyophilized-recombinant StxB was dissolved in 0.5 mL H_2O before centrifugation at 21,000 g (4 °C) for 20 min.

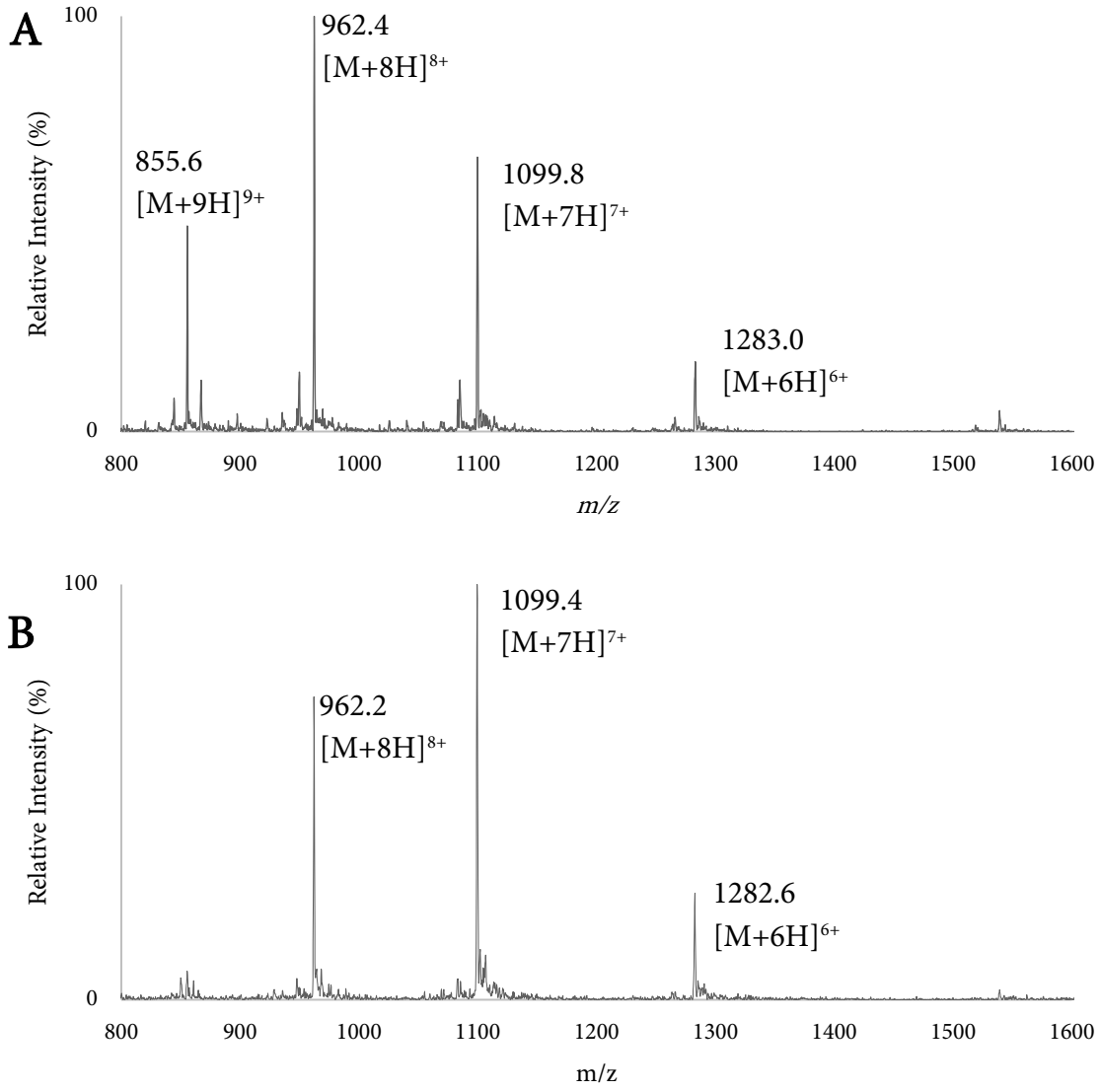

Figure S38: (A) Reduced full-length StxB material before refolding was analyzed using **LC-MS Method B.** Calculated mass = 7690.6 Da, Observed mass = 7691.4 Da. **(B)** Full-length StxB material after 24 h of oxidative dialysis at 37 °C was analyzed using **LC-MS Method B.** Calculated mass = 7688.6 Da, Observed mass = 7689.4 Da (Δ -2 Da indicates successful

formation of disulfide bond). Note the significant shift in the relative intensities of charge state 9+ between reduced and oxidized StxB, suggesting a conformational change of the protein.

Section XIX: Analysis of Folded StxB using High-Resolution Mass Spectrometry

Reversed-phase LC/MS was performed on an Eksigent Ekspert nanoLC 425 system (SciEx) coupled to a Bruker MAXIS ETD II QToF mass spectrometer. Samples were diluted with a 1:1 ratio of sample: 0.1% formic acid in water. A gradient of reversed-phase buffers (Buffer A: 0.2% formic acid in water; Buffer B: 0.2% formic acid in acetonitrile) at a flow rate of 10 µL/min at 60 °C was utilized. The LC run lasted for 74 min with a starting concentration of 15% buffer B increasing to 95% over 53 min. A 10 cm × 1 mm I.D. Waters Acquity UPLC BEH column was employed for chromatographic separation. Raw data files were directly imported into Protein Metrics Intact Mass™ software to generate averaged and deconvoluted spectra. Deconvoluted and calculated masses in **Figure 6A** are monoisotopic masses of reduced StxB.

Section XX: Analysis of Folded StxB using Circular Dichroism

Folded, synthetic StxB in PBS (50 mM $NaPO_4$, 150 mM NaCl, pH 7.4) was concentrated to 22 µM using a Vivacon-500 10 K MWCO filter. Recombinant StxB was diluted to 35 µM with PBS from a 140 µM stock solution. CD spectra of both the synthetic and recombinant StxB were obtained with an AVIV model 410 spectrophotometer. PBS (blank) and samples were added to a 1 mm quartz cuvette and analyzed at 25°C. Wavelength scans from 200-260 nm were performed

at 1 nm resolution with 1 s averaging time in triplicate. Triplicate scans were then averaged and blank subtracted before normalization to mean residue ellipticity ($[\theta] = 100 * \theta / C * l * (n\text{-}1)$, where C is concentration of protein in mM, l is path length in centimeters, and n is the number of residues in the protein).

Section XXI: Analysis of Folded StxB using Analytical Ultracentrifugation

Sedimentation equilibrium studies on recombinant and synthetic Stx1b were performed on an XL-A analytical ultracentrifuge (Beckman Coulter) at 4 ^{0}C. Samples were prepared in 50 mM sodium phosphate pH 7.4 and 150 mM NaCl at three concentrations each (15, 7.5, and 3.75 μM), and equilibrium radial distributions were recorded with UV absorbance (230 nm) at three speeds (12k, 18k, and 24k RPM). The data at all concentrations and speeds were fit globally to a single ideal species using nonlinear least squares distributions via the program HETEROANALYSIS.[10] Buffer density and protein partial specific volumes calculations were performed with SEDNTERP.[11]

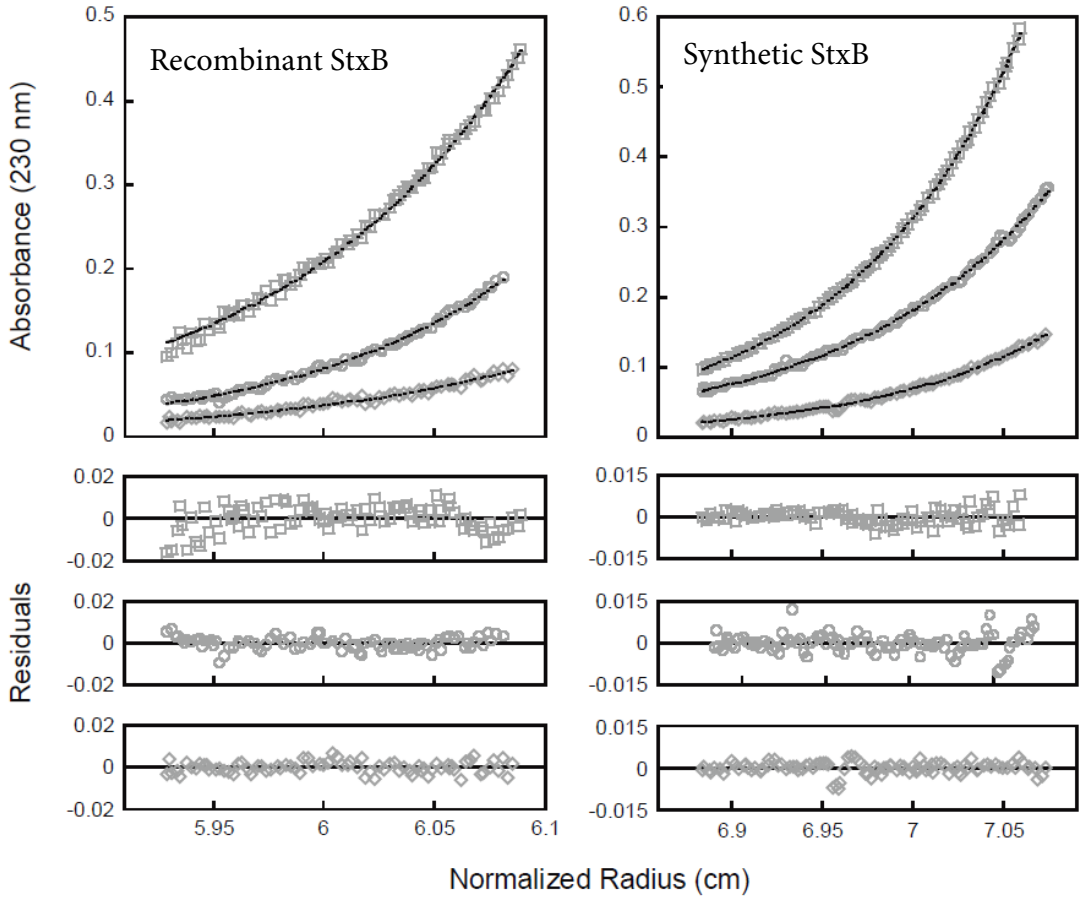

Figure S39: Characterization of recombinant and synthetic StxB by AUC. Representative analytical ultracentrifugation equilibrium data (gray symbols; 18k RPM) and the corresponding global single ideal species fits (solid black lines) for recombinant (left) and synthetic (right) Stx1b. The single ideal species fits correspond to 35,193 Da (Mobs/Mcalc = 4.58) for recombinant Stx1b and 35,076 Da (Mobs/Mcalc = 4.56) for synthetic Stx1b. Fit residuals (the difference between the data and the ideal fit) are indicated in the lower panels for the high, middle, and low concentrations (top to bottom).

Section XXII: Analysis of Folded StxB using Size-Exclusion Chromatography

Folded, synthetic StxB in PBS (22 μM in 50 mM $NaPO_4$, 150 mM NaCl, pH 7.4) and recombinant StxB (12 μM) were analyzed using SEC on an Agilent 1260 with diode array detector and a Phenomenex Yarra 1.8 μm SEC-X150 (4.6 x 150 mm) column . Running buffer for SEC consisted of 50 mM $NaPO_4$, 150 mM NaCl, pH 7.4 with a flow rate of 0.35 mL/min. Molecular weight standards for SEC were thyroglobulin (669 kDa), IgA (300 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and uridine (0.25 kDa).

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