Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

A Glutamic Acid-based Traceless Linker to Address Challenging Chemical Protein Syntheses

Riley J. Giesler[1], Paul Spaltenstein[1], Michael T. Jacobsen[3], Weiliang Xu[1], Mercedes Maqueda[2], Michael S. Kay[1*]

1. Department of Biochemistry, University of Utah School of Medicine, 15 North Medical Drive East, Room 4100, Salt Lake City, Utah 84112-5650, United States of America.

2. Departamento de Microbiología, Universidad de Granada, Avda. Fuentenueva, s/n,18071 Granada, Spain

3. Department of Pediatrics, Division of Diabetes and Endocrinology, Stanford University, Palo Alto, CA 94304, United States of America

*Corresponding Author: kay@biochem.utah.edu

Supplementary Information Contents

Supplementary Figures

Figure # **Page**

Section S1: Materials

Tentagel R RAM resin (0.19 mmol/g) was purchased from Rapp Polymere. 2-chlorotrityl chloride resin (0.77 mmol/g) was purchased from ChemPep. Rink amide ChemMatrix resin (0.4 mmol/g), Fmoc-L-Ala-OH, Fmoc-LAsp(OtBu)-OH, Fmoc-L-Glu(OtBu)-OH, Fmoc-L-Phe-OH, Fmoc-Gly-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Leu-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Pro-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-L-Val-OH, Fmoc-L-Trp(Boc)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-Val-Thr(ΨMe,Mepro)-OH, Fmoc-Gly-Thr(ΨMe,Mepro)-OH, and Fmoc-Leu-Thr(ΨMe,Mepro)-OH were purchased from Gyros Protein Technologies. Fmoc-L-Met-OH and Boc-L-Cys(Trt)-OH were purchased from AAPPTec. Boc-Cys(Trt)-OH was purchased from Sigma Aldrich. TFA-(L)-Thz-OH was synthesized using previously published methods.[1]

Acetic anhydride (99.5%), anhydrous hydrazine (98%), N,N-diisopropylethylamine (DIPEA, ReagentPlus grade), piperidine (ReagentPlus grade), and N-methylmorpholine (NMM, ReagentPlus grade) were purchased from Sigma Aldrich. Fmoc-hydrazine (98%), dimethylformamide (DMF, ACS grade), dichloromethane (DCM, ACS grade), N-methylpyrrolidone (NMP, ≥99.8%), and methanol (MeOH, ACS grade) were purchased from Fisher Scientific. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxidhexafluorophosphate (HATU, 99%) was purchased from Oakwood Chemical.

6-aminohexanoic acid (≥98%) and 4-dimethylamino pyridine (DMAP) were purchased from Sigma Aldrich. Dde-OH was purchased from Novabiochem. N-(3-

Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCl) was purchased from Chem-Impex international. *trans*-2-Butene-1,4-diol was purchased from Toronto research chemicals. Fmoc-(L)-Glu-OtBu was purchased from ChemPep.

Trifluoroacetic acid (TFA, peptide synthesis grade) and anhydrous ethyl ether (ACS grade) were purchased from Fisher Scientific. Triisopropylsilane (TIS, 98%) was purchased from Sigma Aldrich. 1,2-ethanedithiol (EDT, 95%) and ammonium iodide (NH_4I, ≥99%) were purchased from Acros Organics. Trifluoroacetic acid (TFA, HPLC grade) was purchased from Alfa Aesar.

Acetonitrile (ACN, HPLC grade), 0.1% formic acid in water (Optima LC/MS grade), and 0.1% formic acid in acetonitrile (Optima LC/MS grade) were purchased from Fisher Scientific. Optima LC/MS water was purchased from Fisher Scientific. Guanidine hydrochloride (GuHCl, ≥99.5%) was purchased from Thermo Scientific.

Sodium phosphate dibasic heptahydrate (ACS grade) was purchased from AMRESCO. Sodium phosphate monobasic (ACS grade), hydrochloric acid (HCl, ACS plus grade), and sodium nitrite ($NaNO_2$, ACS grade) were purchased from Fisher Scientific. Sodium hydroxide (NaOH, ACS grade) and acetic acid (>99.8%) were purchased from Acros Organics. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl, ≥99%) was purchased from Hampton Research. VA-044 was purchased from Wako. L-glutathione reduced (≥98%), allylpalladium(II) chloride dimer (98%), allyl alcohol (99%), and 4-Mercaptophenylacetic acid (MPAA) (97%) were purchased from Sigma Aldrich. Dithiothreitol (DTT, >99%) was purchased from Goldbio. Palladium(II) acetate (98%) and Triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt (TPPTS, >95%) were

purchased from Sigma Aldrich. SiliaMetS Thiourea functionalized resin (1.3 mmol/g) was purchased from Silicycle. Esprion 300 was purchased from DMV international, carboxymethyl Sepharose matrix was purchased from GE Amersham, and the Vydac 218TP510 C18 Semi-Preparative HPLC Column was purchased from Separation group.

Section S2: Synthesis and Characterization of Fmoc-(L)-Glu(AlHx)-OH

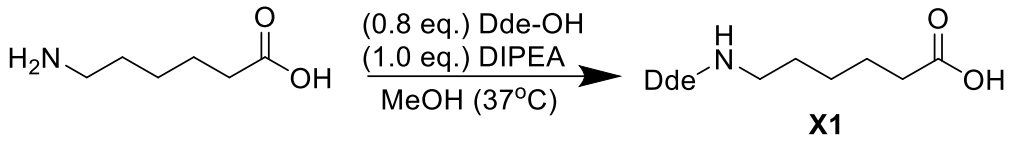


Figure S1. Preparation of 6-((1-(4,4-dimethyl-2,6 dioxocyclohexylidene)ethyl)amino)hexanoic acid (6-(Dde)aminohexanoic acid) (**X1**).

6-aminohexanoic acid (18.7 g, 142.9 mmol) was suspended in 250 mL of methanol and DIPEA (18.4 g, 142.9 mmol) and stirred at room temperature. 2-Acetyldimedone (Dde-OH) (20 g, 109.9 mmol) was added, and the reaction was warmed to 37 ºC and stirred overnight. The methanol was removed under reduced pressure, and the mixture was suspended in water and acidified with HCl (conc.) to pH 3-4. 6-(Dde-amino)hexanoic acid (**X1**) was extracted with methylene chloride (5x, 50 mL) and dried with Na_2SO_4. The product was concentrated under reduced pressure to produce a white solid and was used without further purification.

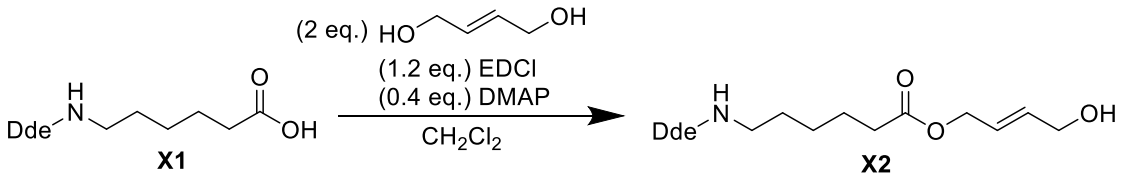


Figure S2. Preparation of (*E*)-4-hydroxybut-2-en-1-yl 6-((1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)amino)hexanoate (**X2**).

4.52 g (15.34 mmol) of **X1** was dissolved in 25 mL of DCM, and EDCl (3.5 g, 18.4 mmol) and DMAP (749 mg, 6.1 mmol) were added. In a separate flask, (*E*)-2-Butene-1,4-diol (2.7 g, 30.7 mmol) was suspended in 25 mL of DCM. The activated **X1** solution was slowly added to the diol solution dropwise, and the reaction was stirred at room temperature for 3 hours. After the reaction was complete, the product was concentrated under reduced pressure and directly purified by flash chromatography with a methylene chloride/methanol mixture (1% MeOH up to 4% MeOH) to afford 3.9 g (10.9 mmol) of the product ((*E*)-4-hydroxybut-2-en-1-yl 6-(Dde)aminohexanoate) (**X2**) in 55% yield.

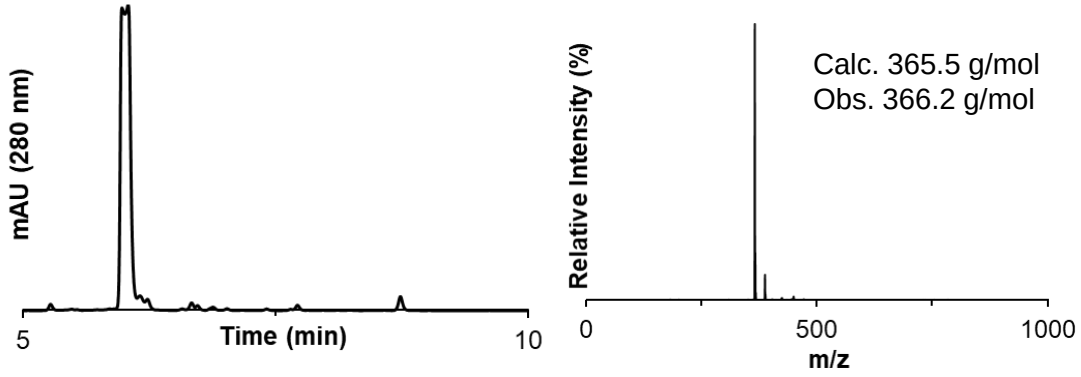


Figure S3. LC/MS analysis of **X2** using **LC/MS method C.**

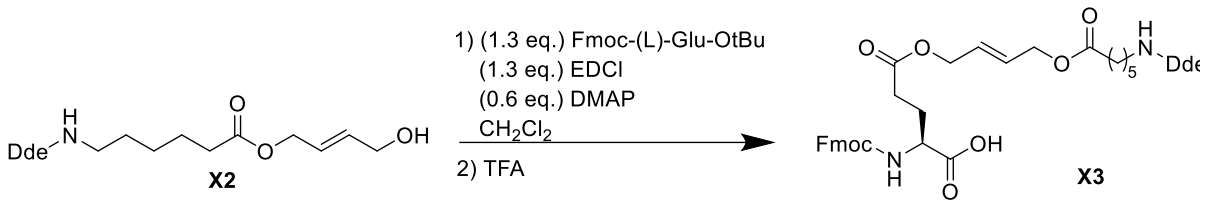


Figure S4. Preparation of Fmoc-(L)-Glu((*E*)-4-hydroxybut-2-en-1-yl 6-((1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)amino)hexanoate)-OH (**X3**).

Fmoc-L-Glu-OtBu (4.7 g, 10.9 mmol) was dissolved in 35 mL of methylene chloride, and EDCl (2.1 g, 10.9 mmol) and 798 mg of DMAP (6.54 mmol) were added. 3.1 g (8.4 mmol) of **X2** were added, and the reaction was stirred at room temperature for 3 hours.After the reaction was complete, the solvent was removed under reduced pressure. 10 mL of TFA were then added, and the reaction was stirred for another 3 hours. After evaporation of the TFA, the final compound was purified by flash chromatography with a DCM/methanol mixture (1% MeOH to 8% MeOH) to afford 3.6 g (5.0 mmol) of Fmoc-L-Glu(AlHx)-OH (**X3**) in 59% yield.

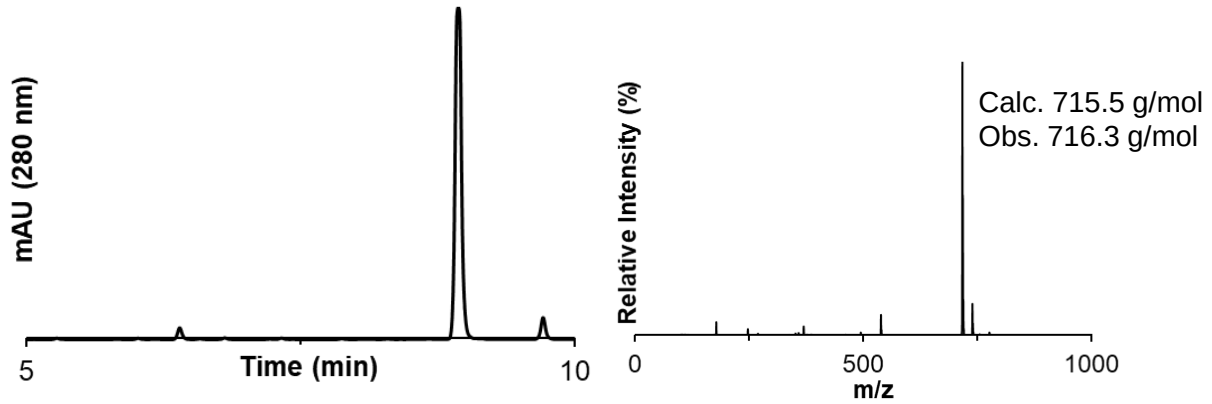


Figure S5. LC/MS analysis of **X3** using **LC/MS method C.**

^{1}H NMR (600 MHz, $CDCl_3$): δ = 13.32 (s, 1H, NH_{Dde}), 11.26 (s, 1H, $COOH_{Glu}$), 7.76 (d, 2H, HAr_{Fmoc}), 7.61 (d, 2H, HAr_{Fmoc}), 7.39 (t, 2H, HAr_{Fmoc}), 7.30 (t, 2H, HAr_{Fmoc}), 5.82 (ovlp, 2H, CCH_{AlHx}), 5.67 (d, 1H, NH_{Glu}), 4.58 (s, 4H, CH_2O_{AlHx}), 4.41 (m, 1H, $CH_{Glu(Ca)}$),

4.39 (d, 2H, CH_{2Fmoc}), 4.22 (t, 1H, CH_{Fmoc}), 3.42 (dd, 2H, CH_2N_{AlHx}), 2.55 (s, 3H, CH_{3Dde}), 2.47-2.43 (m, 2H, $CH_{2AlHx(C2)}$), 2.43 (s, 4H, CH_{2Dde}) 2.32 (t, 2H, $CH_{2Glu(Cg)}$), 2.28-2.04 (m, 2H, $CH_{2Glu(Cb)}$), 1.69 (m, 4H, $CH_{2AlHx(C3,5)}$), 1.41 (m, 2H, $CH_{2AlHx(C4)}$), 1.04 (s, 6H, CH_{3Dde}).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 198.9, 174.7, 174.2, 173.1, 172.5, 156.2, 143.8, 143.7, 141.3, 128.2, 127.9, 127.8, 127.1, 125.1, 120.0, 107.5, 67.1, 64.1, 63.8, 53.2, 51.8, 47.1, 43.7, 33.8, 30. 3, 28.5, 28.1, 27.5, 26.2, 24.3, 18.6

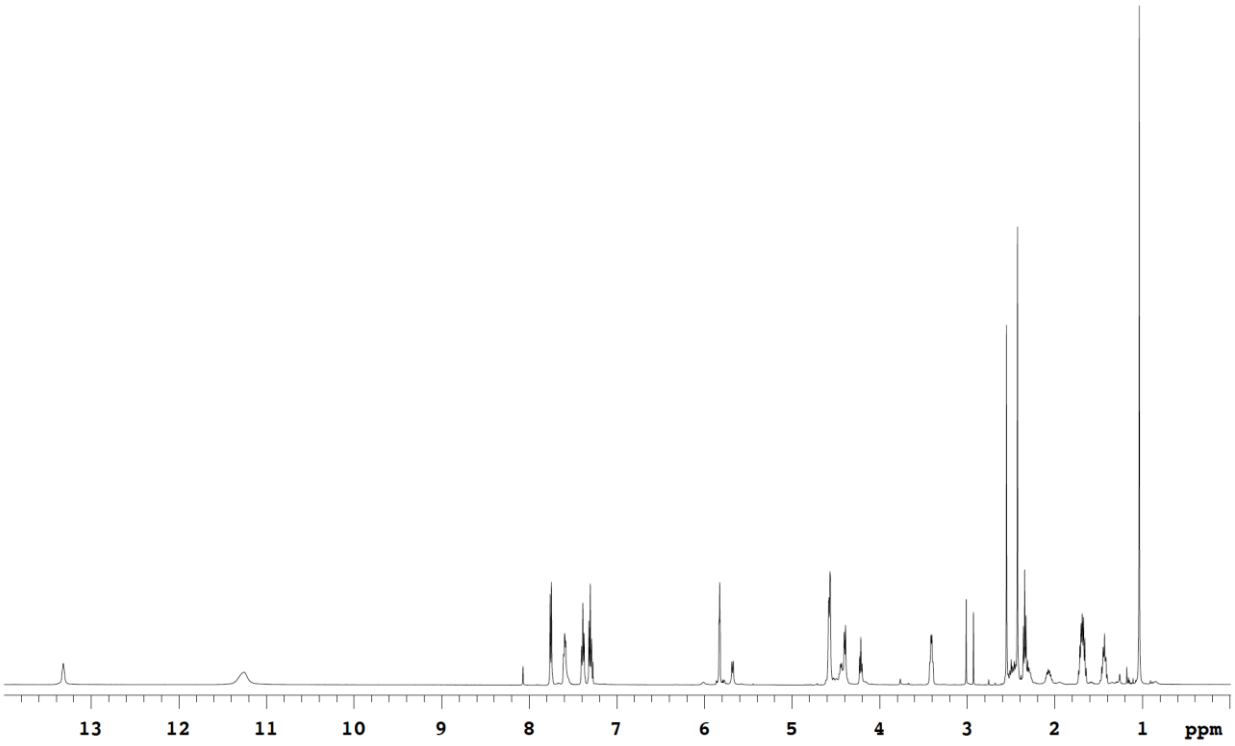


Figure S6. ^{1}H NMR spectrum of **X3.**

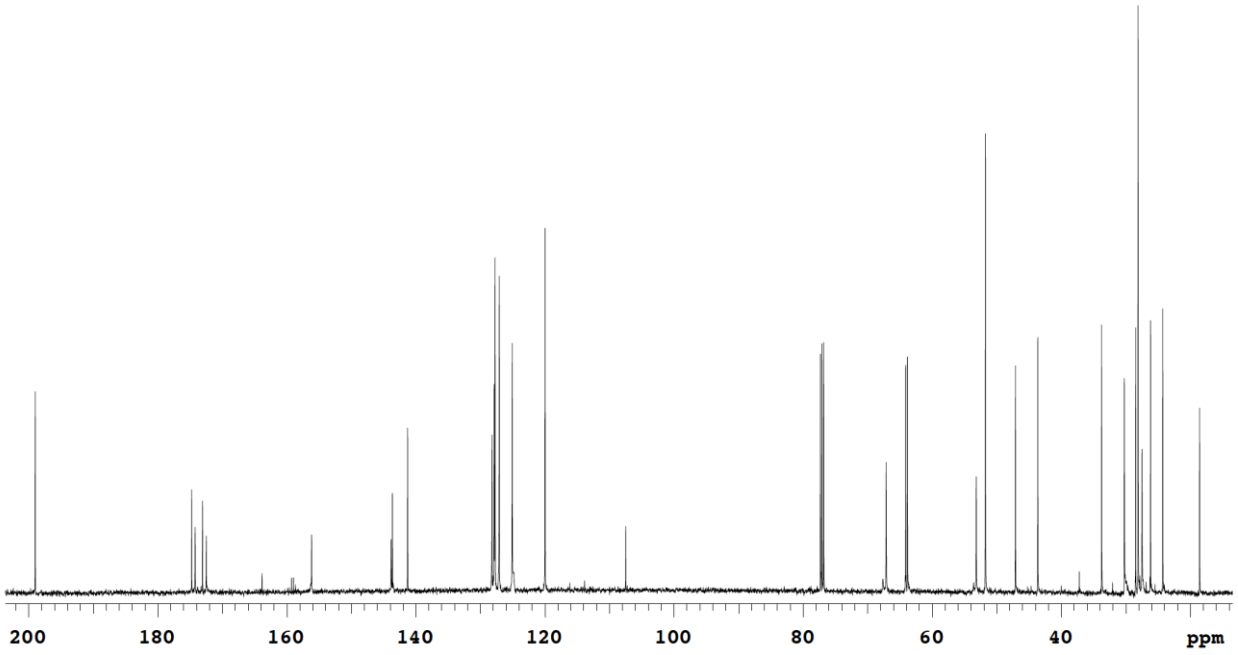


Figure S7. ^{13}C NMR spectrum of **X3.**

Section S3: 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 bubbling 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 hour on rotisserie at room temperature. Unreacted groups were capped with 20 mL of 17:2:1 DCM:MeOH:DIPEA for 5 min. 2-Chlorotrityl chloride resin was converted to 2-chlorotrityl Fmoc-hydrazine according to published protocol to generate C-terminal hydrazides.[2, 3] N-terminal capping was achieved through the addition of 1 mL acetic anhydride and 1 mL 0.6 M NMM per 30 μmol resin. After completion of syntheses, peptide resins were thoroughly washed with DCM and dried under vacuum.

Section S4: Procedure for Synthesizing AlHx-Modified Peptides

Fmoc-L-Glu(AlHx)-OH was incorporated into peptides using the standard methods outlined above. After the peptide N-terminus was protected by either Ac- or Boc-, the Dde group was removed with three treatments of 1.7 mL of 5% hydrazine in DMF and 0.3 mL of allyl alcohol for 5 min each. The resin was washed 5 times with 2 mL of DMF and DCM. Lys_6 tags were added using standard coupling methods on the Prelude X instrument.

Section S5: General Methods for Peptide Cleavage

Cleavage of peptide resins was achieved by 3 hours 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, 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 4 ºC and 5,000 g for 10 min. The supernatant was decanted while pellets were washed twice with ether before being dried under vacuum.

Section S6: General Methods for HPLC and LC/MS

LC/MS Methods:

0.1% formic acid in water (Buffer A) and 0.1% formic acid in ACN (Buffer B) were used as mobile phases for LC/MS analyses. Mass spectra were obtained 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). Unless otherwise noted, observed masses were manually calculated using the charge states from averaged scans across the major ion signal and corresponding UV peak.

Method	Column	Gradient and Flow Rate
A	Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm)	5-90% B over 7 min 0.75 mL/min
B	Phenomenex Aeris widepore 3.6 µm C4 (200 Å, 2.1 x 50 mm)	5-90% B over 7 min 0.5 mL/min
C	Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm)	15-95% B over 10 min 0.8 mL/min

Analytical methods:

0.1% TFA in water (Buffer A) and 0.1% TFA in 90% ACN (Buffer B) were used as mobile phases for analytical HPLC analyses. Traces were collected on an Agilent 1260 Infinity II instrument at A_{214}. Specific analytical methods are described below

Method	Column	Gradient and Flow Rate
A	Phenomenex Jupiter 4 µm C12 (90 Å, 150 x 4.6 mm)	10-90% B over 25 min 1 mL/min
B	Phenomenex Jupiter 4 µm C12 (90 Å, 150 x 4.6 mm)	30-70% B over 25 min 1 mL/min
C	Phenomenex Jupiter 5 µm C4 (300 Å, 150 x 4.6 mm)	10-90% B over 25 min 1 mL/min

Purification methods:

0.1% TFA in water (Buffer A) and 0.1% TFA in 90% ACN (Buffer B) were used as mobile phases for preparative HPLC purifications. Peptide purifications were performed on an Agilent 1260 Infinity II LC system or Agilent 1260 Infinity II preparative system. Specific purification methods are described below:

Method	Column	Gradient and Flow Rate
A	Phenomenex Jupiter 4 µm C12 (90 Å, 250 x 21.20 mm)	40-46% B over 25 min 20 mL/min
B	Phenomenex Jupiter 5 µm C4 (300 Å, 250 x 10 mm)	40-60% B over 35 min 4 mL/min
C	Phenomenex Jupiter 4 µm C12 (90 Å, 250 x 21.20 mm)	33-37% B over 25 min 15 mL/min
D	Phenomenex Jupiter 5 µm C4 (300 Å, 250 x 10 mm)	40-85% B over 40 min 5 mL/min
E	Phenomenex Jupiter 5 µm C4 (300 Å, 250 x 10 mm)	30-90% B over 25 min 4 mL/min

Section S7: General Methods for NCL and Desulfurization

Native chemical ligation reactions were performed according to standard methods using the thiol additive MPAA and TCEP reducing agent.[2, 4, 5] All ligation reactions employed peptide hydrazide method, whereby peptides were dissolved and activated (conversion of hydrazide to acyl azide) in activation buffer (6 M GuHCl, 100 mM phosphate, pH 3) for 20 min at -20 ºC by addition of freshly prepared 15 eq sodium nitrite. Following activation, a solution containing freshly prepared MPAA pH 7 in ligation buffer (6 M GuHCl, 100 mM phosphate, pH 7) was added, and the final pH was adjusted to 7 to initiate thiolysis and ligation reaction. Upon completion (based on analytical HPLC and

LC/MS), reactions were treated with freshly prepared 150 mM TCEP in 6 M GuHCl for 10 min, spun at 5000 g, and the supernatant was purified by preparative HPLC.

Desulfurization was performed using established metal-free, radical-mediated procedures.[6] Using degassed desulfurization buffer (6 M GuHCl, 100 mM phosphate, pH 6.5), two stock solutions were prepared: solution A (120 mM VA-044 and 240 mM reduced GSH) and solution B (500 mM TCEP). Peptides were dissolved in a minimal amount of degassed desulfurization buffer, and equal volumes of solution A and B were added to the peptide. The final pH was adjusted to 6.5, and the reactions were stirred at 37 ºC until desulfurization was complete. The reaction was diluted with 5% AcOH, centrifuged at 5000 g, and the solution was purified by preparative HPLC.

Section S8: Original Procedure for Removing AlHx from Peptides

The AlHx linker was removed with 25 molar equivalents of $[Pd(allyl)Cl]_2$ and reduced glutathione. Peptides were dissolved to ~1 mM in sparged ligation buffer at pH 7. 25 molar equivalents of $[Pd(allyl)Cl]_2$ and reduced GSH were dissolved in a small amount (25-100 µL) of sparged buffer and added to the peptide solution. The pH was adjusted to 8, and the reaction was mixed at 37 ºC. After the reaction was complete, 60 molar equivalents of DTT were added to reduce and precipitate the Pd, the sample was centrifuged at 14,000 rpm for 10 min, and the supernatant was purified via RP-HPLC.

Section S9: New Procedure for Removing AlHx from Peptides

To remove the AlHx linker with the new Pd-catalyzed method, peptides were dissolved in degassed ligation buffer to ~1 mM at pH 7. A stock solution of $Pd(TPPTS)_4$ (~100 mM) was prepared as previously described.[7] Briefly, a 850 mM TPPTS solution was

prepared in ddH_2O. A second solution of 188 mM $Pd(OAc)_2$ was prepared in degassed DMF. Equal volumes of the $Pd(OAc)_2$ and TPPTS solutions were mixed together and vortexed for 30 s to create the ~100 mM $Pd(TPPTS)_4$ stock solution. $Pd(TPPTS)_4$ was added to the peptide solution to a final concentration of 10 mM Pd and 1 mM peptide. After stirring at room temperature for 15-30 min, the reaction was added to 15 molar equivalents of the thiourea resin (based on the loading density of the resin). After stirring for 5 min, the reaction was centrifuged at 14,000 rpm for 10 min to pellet the resin. The supernatant was diluted with buffer A and analyzed or purified by HPLC and LC/MS.

Section S10: Synthesis and Purification of C20E Model Peptide With AlHx-K_6

C20-K9E (referred to as C20E, Ac-DWTKNITD**X**IDQIIHDFVDK-NH2, where X is Glu(AlHx)) was prepared on TentaGel R RAM resin to generate a peptide with C-terminal amide. The N-terminus was capped using acetic anhydride, Dde was removed from Glu(AlHx), and Lys_6 was added using standard SPPS conditions as outlined above. After TFA cleavage and global deprotection, 113 mg of crude C20E was dissolved in 40 mL of 10% ACN/H_2O (0.1% TFA) and purified with **purification method A**. HPLC purification yielded 50.3 mg of the pure peptide which was used for further stability and removal studies. The crude and purified peptides were analyzed with **analytical method A and LCMS method A**.

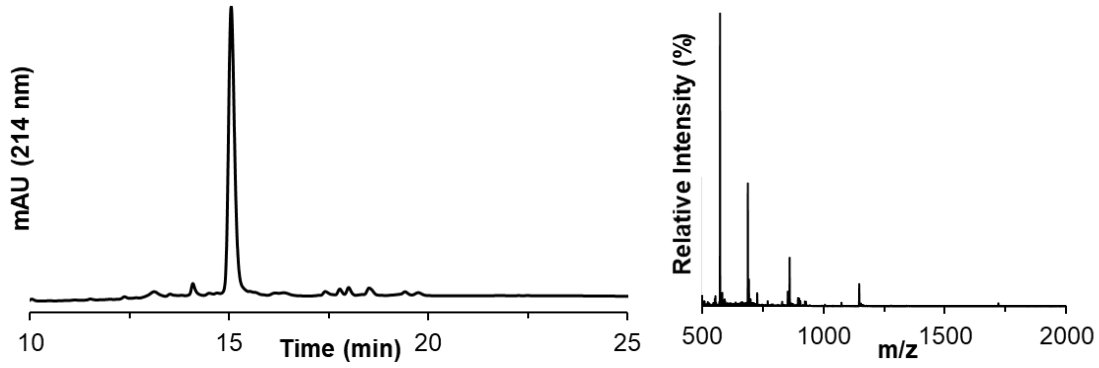


Figure S8. Analytical HPLC and LC/MS analysis of crude Ac-C20E(AlHx-K_6)-NH_2.

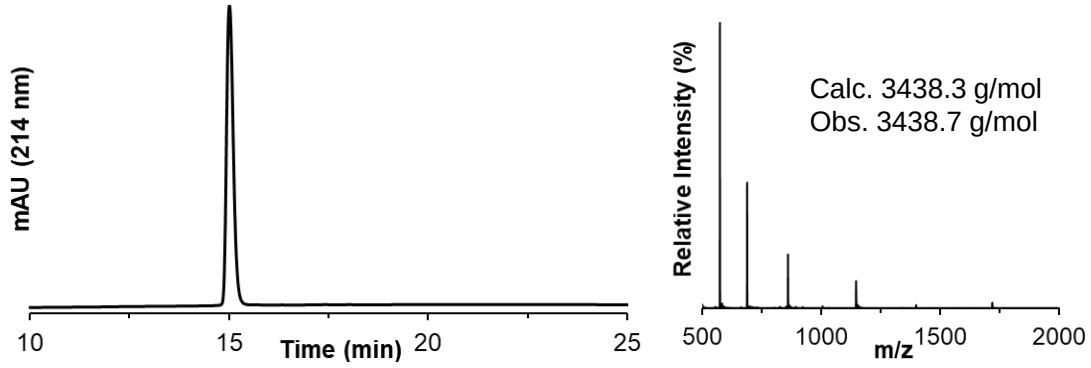


Figure S9. Analytical HPLC and LC/MS analysis of pure Ac-C20E(AlHx-K_6)-NH_2.

Section S11: Cleavage of AlHx-K_6 from C20E Model Peptide (Original Procedure)

To evaluate how quickly the AlHx linker was removed, purified C20E(AlHx-K6) was dissolved in 500 µL of degassed ligation buffer to 1 mM. A solution of $[Pd(allyl)Cl]_2$ (4.56 mg, 12.5 µmol) and reduced GSH (3.85 mg, 12.5 µmol) were dissolved in 25 µL (500 mM) of degassed ligation buffer and briefly sonicated. The Pd solution was added to the peptide solution for a final reaction volume of 525 µL, and the pH was adjusted to 8. To monitor the reaction, 100 µL of the peptide solution was mixed with 12 µL of a 500 mM DTT (60 molar equivalents) solution in ddH_2O, and the reaction was stirred for 10 min to precipitate the Pd. The reaction was centrifuged at 14,000 rpm for 10 min to pellet the

Pd-DTT complex. The supernatant was diluted with buffer A and analyzed directly by HPLC and LC/MS with **analytical method A** and **LC/MS method A**.

Section S12: Cleavage of AlHx from C20E Model Peptide (New Procedure)

To evaluate the new Pd-catalyzed removal method, purified C20E(AlHx-K6) was dissolved in 630 µL of degassed ligation buffer to 1.1 mM. A stock solution of $Pd(TPPTS)_4$ (~100 mM) was prepared as described above. 70 µL of $Pd(TPPTS)_4$ was added to the C20E(AlHx-K_6) solution to a final concentration of 10 mM Pd and 1 mM peptide (final reaction volume of 700 µL). To monitor the reaction, 100 µL was mixed with 12 mg of thiourea resin (~15 molar equivalents to C20E). After stirring for 5 min, the reaction was centrifuged at 14,000 rpm for 10 min to pellet the resin. The supernatant was diluted with buffer A and analyzed directly by HPLC and LC/MS with **analytical method A** and **LC/MS method A**.

Section S13: Studies on AlHx-K_6 Stability in C20E Model Peptide

To determine the stability of the AlHx linker in common CPS conditions, C20E(AlHx-K_6) was dissolved to 1 mM in the following conditions, incubated at room temperature over 48 hours, and monitored by HPLC with **analytical method B**.

A: 50% acetonitrile/water + 0.1% TFA (HPLC buffer)

B: 6 M GuHCl, 100 mM PO_4, pH 3 (activation buffer)

C: 6 M GuHCl, 100 mM PO_4, pH 7 (ligation buffer)

D: 6 M GuHCl, 100 mM PO_4, 200 mM $MeONH_2$, pH 4 (Thz removal conditions)

E: 6 M GuHCl, 100 mM PO_4, 200 mM MPAA, 50 mM TCEP, pH 7 (NCL conditions)

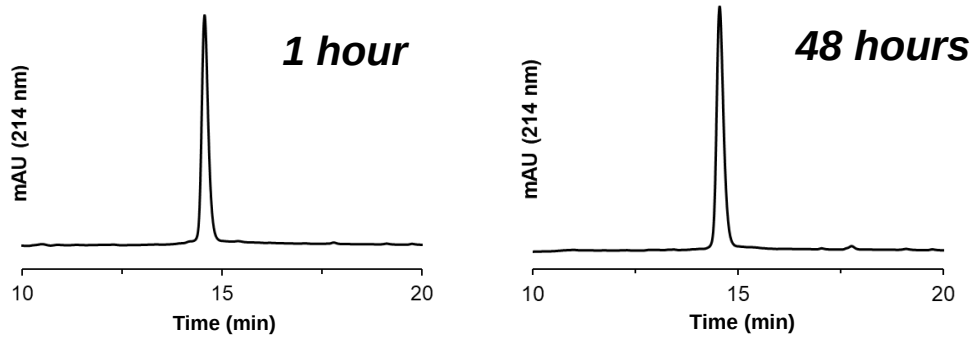


Figure S10. HPLC analysis of C20E(AlHx-K_6) stability in 50% ACN/H_2O + 0.1% TFA over 48 hours.

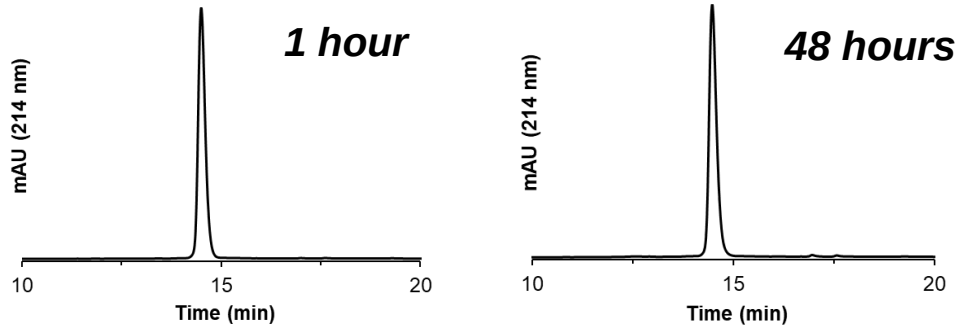


Figure S11. HPLC analysis of C20E(AlHx-K_6) stability in activation buffer over 48 hours.

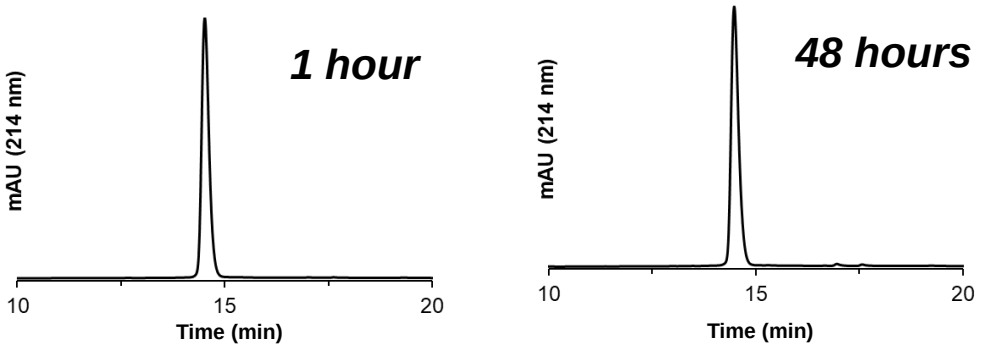


Figure S12. HPLC analysis of C20E(AlHx-K_6) stability in ligation buffer over 48 hours.

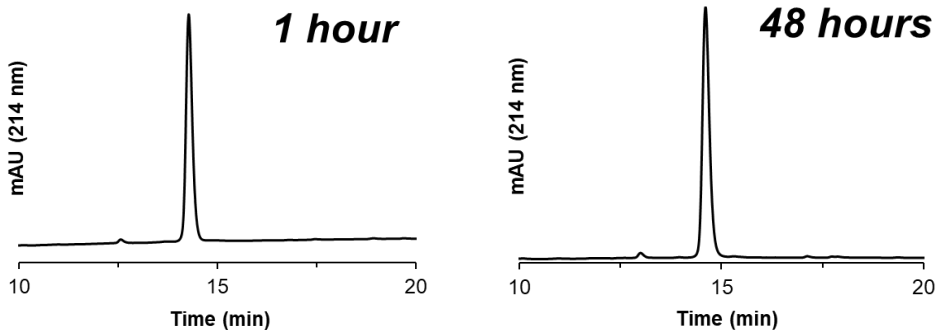


Figure S13. HPLC analysis of C20E(AlHx-K_6) stability in Thz removal conditions over 48 hours.

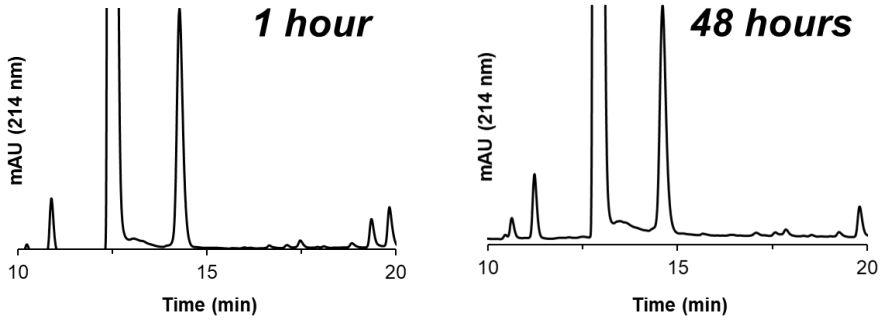


Figure S14. HPLC analysis of C20E(AlHx-K_6) stability in NCL removal conditions over 48 hours The peak at ~12.5 min corresponds to MPAA.

Section S14: Synthesis of AS-48 – Initial Peptide Segments

AS-48(10-44) H-CVAGTVLNVV**X**AGGWVTTIVSILTAVGSGGLSLLA-$NHNH_2$, where **X**=E(AlHx-K_6), 319 mg of crude peptide was dissolved in 55 mL of 20% ACN/H_2O (0.1% TFA) and purified with **purification method B**. HPLC purification yielded 32.9 mg of pure peptide. The crude and pure peptides were analyzed by HPLC and LC/MS with **analytical method C** and **LC/MS method B**.

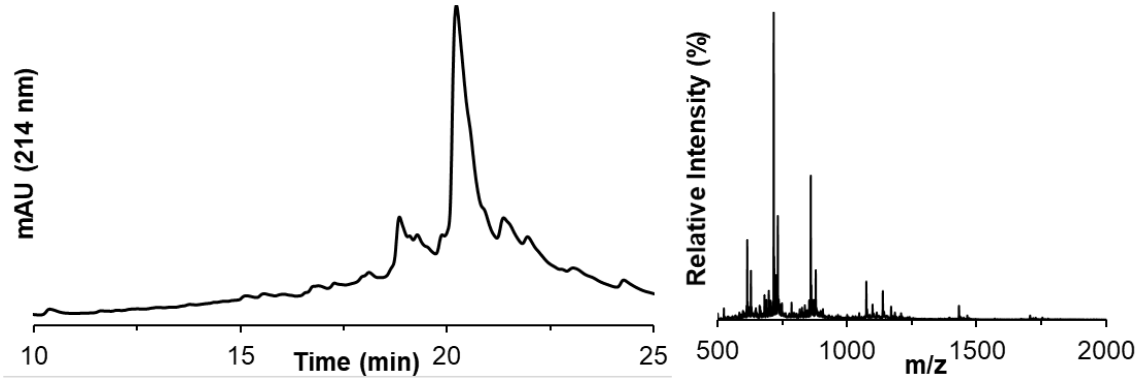


Figure S15. HPLC and LC/MS analysis of crude AS-48(10-44)(AlHx-K_6)-$NHNH_2$.

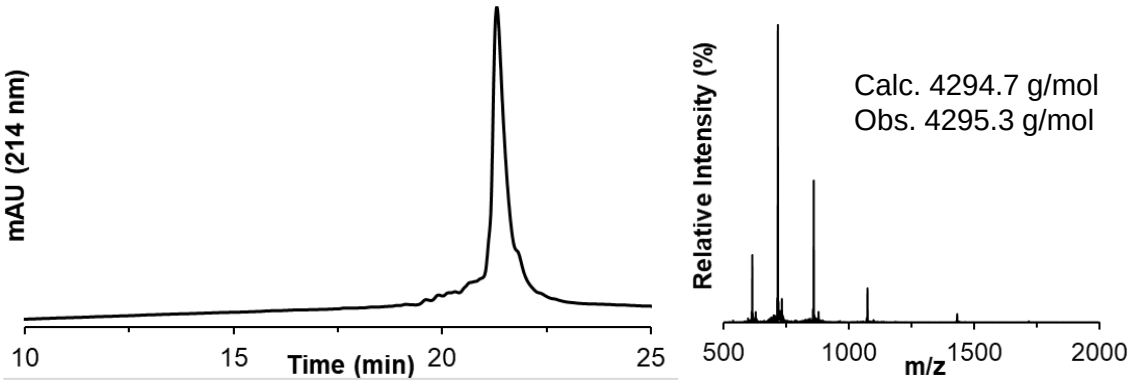


Figure S16. HPLC and LC/MS analysis of pure AS-48(10-44)(AlHx-K_6)-$NHNH_2$.

AS-48(45-70,1-9) (Tfa-Thz-AGRESIKAYLKKEIKKKGKRAVIAWMAKEFGIPA-$NHNH_2$)
188 mg of crude peptide was dissolved in 40 mL of 10% ACN/H_2O (0.1% TFA) and purified with **purification method C**. HPLC purification yielded 64.1 mg of pure peptide. The crude and pure peptides were analyzed by HPLC and LC/MS with **analytical method C** and **LC/MS method B**.

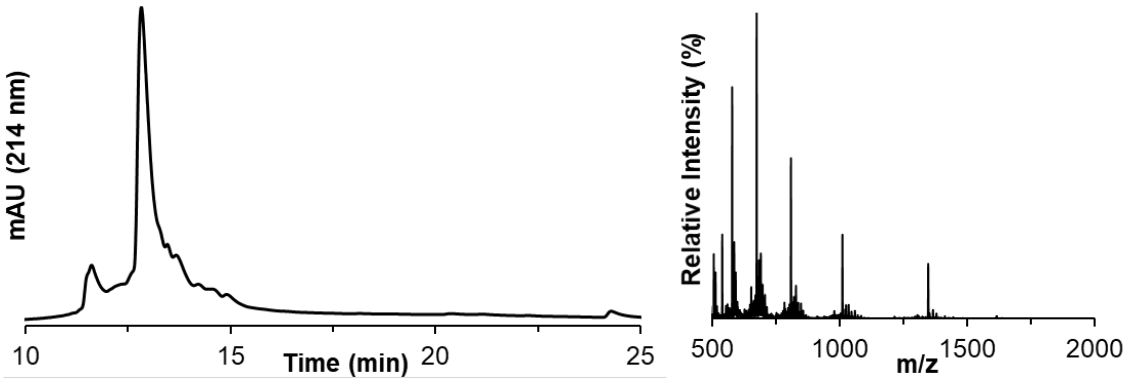


Figure S17. HPLC and LC/MS analysis of crude Tfa-Thz-AS-48(45-70,1-9)(AlHx-K_6)-$NHNH_2$.

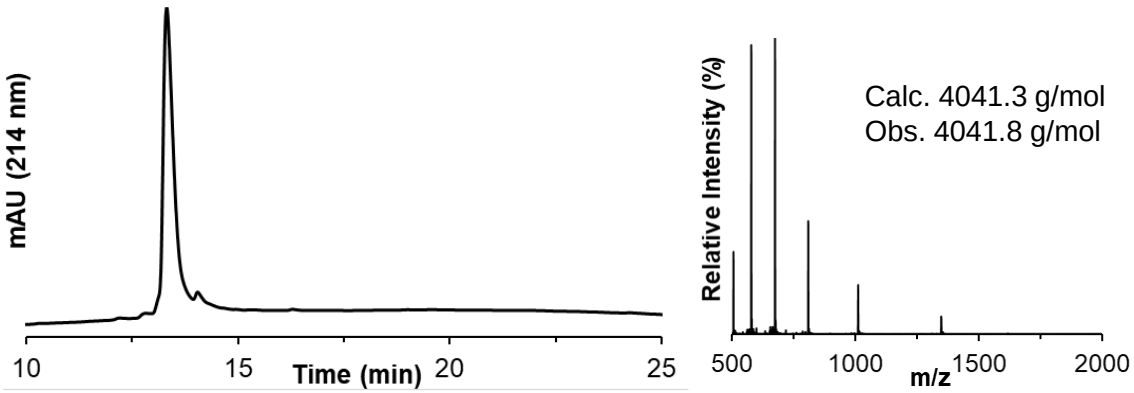
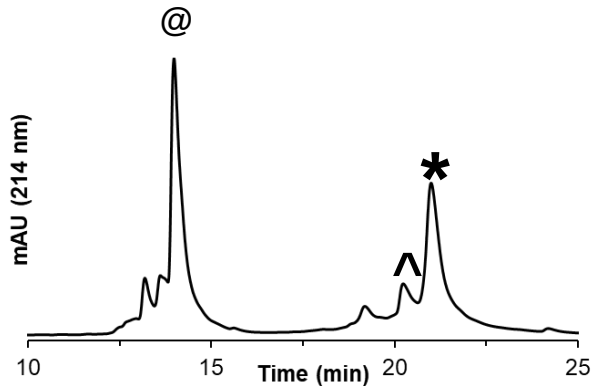


Figure S18. HPLC and LC/MS analysis of pure Tfa-Thz-AS-48(45-70,1-9)(AlHx-K_6)-$NHNH_2$.

Section S15: Synthesis of AS-48 – Ligation Products and Full-Length Protein

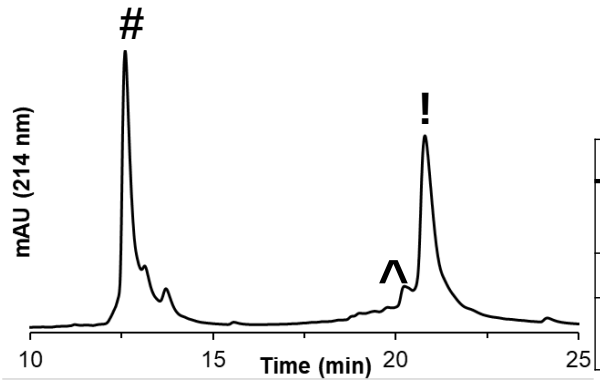
Native chemical ligation between AS-48(10-44) and AS-48(45-70,1-9) was performed using the peptide hydrazide method. 26.3 mg of AS-48(45-70,1-9) (6.5 µmol) was

dissolved in 3 mL (2.1 mM) of activation buffer, and the pH was adjusted to 3. The peptide was cooled to -20° C for 10 min, and 195 µL of 500 mM $NaNO_2$ pH 3 was added to 30.6 mM (15 eq. to AS-48(45-70,1-9)). The peptide was incubated at -20° C for 20 min. 65.6 mg of MPAA (390.6 µmol, 50 eq. to AS-48(45-70,1-9)) was dissolved in 1 mL of ligation buffer, and the pH was adjusted to ~6. The MPAA solution was added to the activated peptide solution, and the resulting solution was stirred at room temperature for 5 min. 20 mg of lyophilized AS-48(10-44) peptide (4.66 µmol) was directly added to the solution, and the pH was adjusted to ~7. After 15 min, 450 µL of a 300 mM TCEP pH 7 solution was added. After 24 h, the pH was adjusted to 8 and rotated at room temperature for 1 h. The pH was then adjusted to 5, and 83 mg of methoxyamine hydrochloride was added and immediately sonicated. The pH was adjusted to 4, and the reaction was stirred for 4 h. Finally, the ligation was reduced with 150 mM TCEP pH 7 and diluted to 3 M GuHCl with 5% AcOH in water. After spinning the sample at 14,000 rpm for 15 min, the supernatant was purified by RP-HPLC with **purification method D** to yield 6.8 mg of the full-length linear AS-48(1-70) in 17.9% yield. The ligation reaction and pure peptide was analyzed by HPLC and LC/MS with **analytical method C** and **LC/MS method B**.



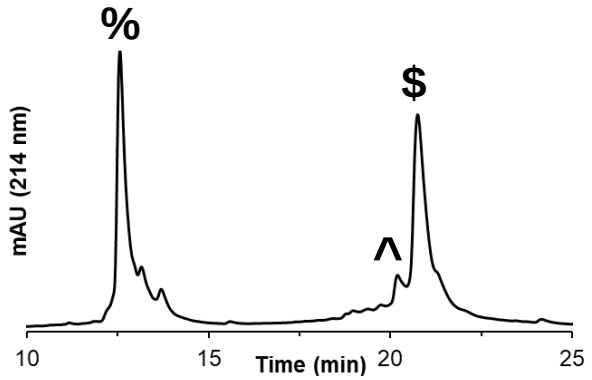
Symbol	Peptide
@	AS-48(45-70,1-9) ***(TFA-Thz)***
^	AS-48(10-44)(AlHx-K_6)
*	AS-48(45-70,1-44)(AlHx-K_6) ***(TFA-Thz)***

Figure S19. Ligation between AS-48(45-70,1-9) and AS-48(10-44)(AlHx-K_6) after 1 hour.



Symbol	Peptide
#	AS-48(45-70,1-9) ***(Thz)***
^	AS-48(10-44)(AlHx-K_6)
!	AS-48(45-70,1-44)(AlHx-K_6) ***(Thz)***

Figure S20. Ligation between AS-48(45-70,1-9) and AS-48(10-44)(AlHx-K_6) after 24 hours. At this point, the TFA adduct had hydrolyzed from the Thz group.



Symbol	Peptide
%	AS-48(45-70,1-9) ***(No TFA-Thz)***
^	AS-48(10-44)(AlHx-K_6)
$	AS-48(45-70,1-44)(AlHx-K_6) ***(No TFA-Thz)***

Figure S21. Thz removal of the ligated AS-48(45-70,1-44)(AlHx-K_6) after 3 hours.

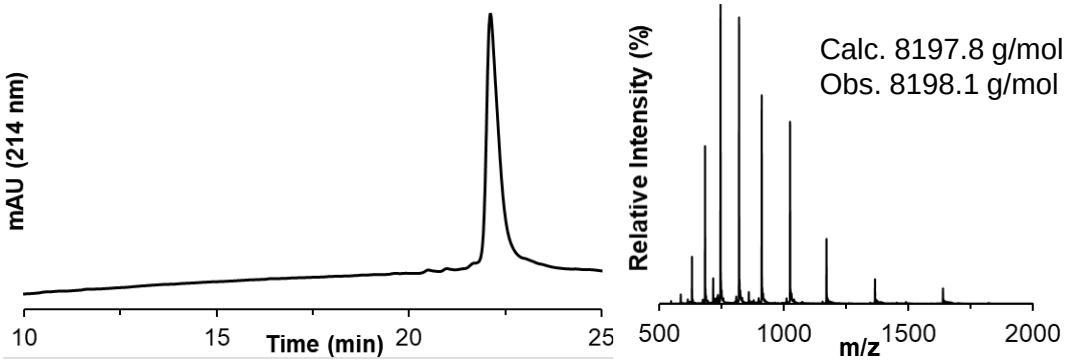


Figure S22. HPLC and LC/MS analysis of pure AS-48(45-70,10-44)(AlHx-K_6)-$NHNH_2$.

To cyclize AS-48(1-70), a second NCL reaction was performed, also through the peptide hydrazide method. 15.6 mg (1.91 µmol) of AS-48(1-70) was dissolved in 5 mL of activation buffer, and the pH was adjusted to 3. The peptide was cooled to -20° C for 10 min and 38.2 µL of 500 mM $NaNO_2$ pH 3 was added to a concentration of 5.7 mM (19.1 µmol, 10 eq. to AS-48). 16.1 mg of MPAA (95.5 µmol, 50 eq. to AS-48) was dissolved in 4 mL of ligation buffer, and the pH was adjusted to 6. The MPAA solution was added to the activated peptide solution, and the pH was adjusted to 7. After 15 min, 760 µL of a 250 mM TCEP solution (0.19 mmol) pH 7 was added for a final reaction volume of ~10 mL. The NCL reaction was complete after 60 min. The reaction was then reduced with 150 mM TCEP pH 7 and diluted to 3 M GuHCl with 5% AcOH in H_2O. After spinning the sample at 14,000 rpm for 15 min, the supernatant was purified by RP-HPLC by **purification method D** to yield 5.8 mg of the cyclized AS-48(1-70) in 37% yield. The ligation reaction and pure peptide were analyzed by HPLC and LC/MS with **analytical method C** and **LC/MS method B**.

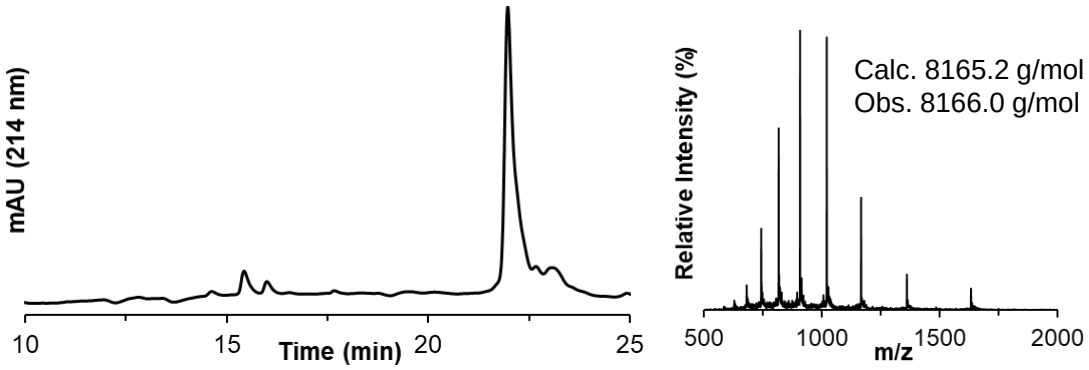


Figure S23. Intramolecular ligation of AS-48(45-70,10-44)(AlHx-K_6)-$NHNH_2$ after 1 hour. Note the shown mass spectrum corresponds to only the main peak, not the entire reaction sample.

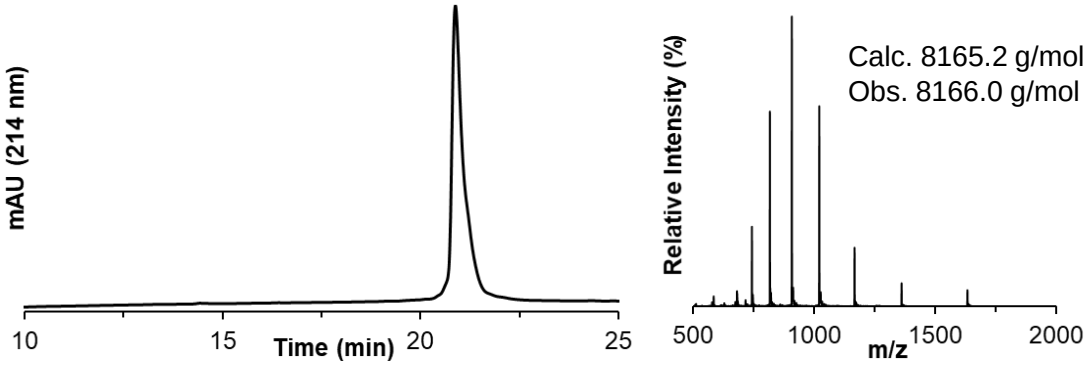


Figure S24. HPLC and LC/MS analysis of pure cyclized AS-48(1-70)(AlHx-K_6).

Next, 10 mg of cyclized AS-48(1-70) (1.2 µmol) was dissolved in 600 µL of a 120 mM VA-044 and 240 mM reduced glutathione solution in desulfurization buffer. 600 µL of a 500 mM TCEP solution was added, and the final pH was adjusted to 6.5 (final concentrations were 1 mM AS-48, 60 mM VA-044, 120 mM GSH, and 250 mM TCEP). The reaction was stirred at 37 ºC for 4 h and monitored by analytical HPLC and LCMS with **analytical method C** and **LC/MS method B**.

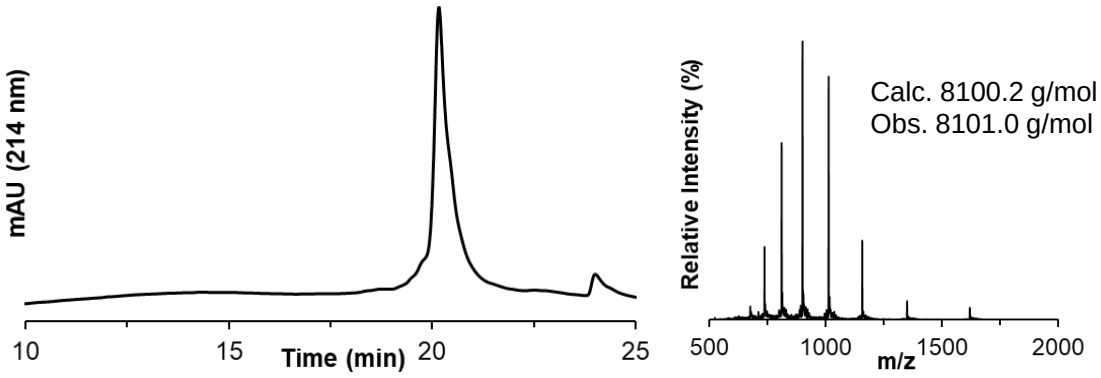


Figure S25. Desulfurization of cyclized AS-48(1-70)(AlHx-K_6) after 4 hours. The AlHx-K_6 was removed in a one-pot fashion without purifying the intermediate. Note the shown mass spectrum corresponds to only the main peak, not the entire reaction sample.

After the reaction was complete, 30 µmol of $[Pd(allyl)Cl]_2$ (11 mg) and reduced GSH (9 mg) were dissolved in 100 µL of ligation buffer and briefly sonicated. The Pd solution was added directly into the AS-48 solution, and the pH was adjusted to 8 (final concentrations were ~0.9 mM AS-48, 23 mM Pd, 23 mM GSH). The reaction was stirred at 37 ºC for 90 min. After the reaction was complete, 72 µmol of DTT powder (11 mg) was added to the reaction to deactivate and precipitate the Pd metal. The reaction was stirred for 10 min, centrifuged at 14,000 rpm to pellet the DTT-Pd complex, and the

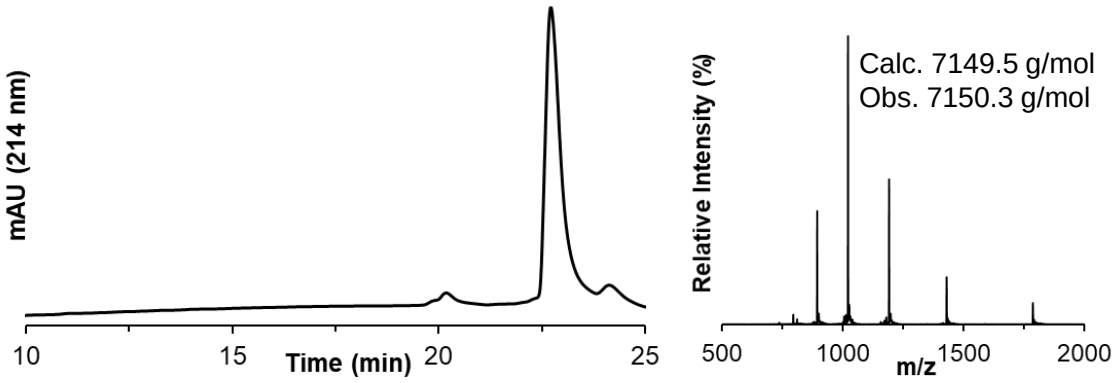


Figure S26. AlHx-K_6 removal from cyclized AS-48(1-70) after 90 min. Note the shown mass spectrum corresponds to only the main peak, not the entire reaction sample.

supernatant purified by HPLC with **purification method E** to yield 1.2 mg (amount determined by UV absorbance) of the final native AS-48 protein in 13.9% yield.

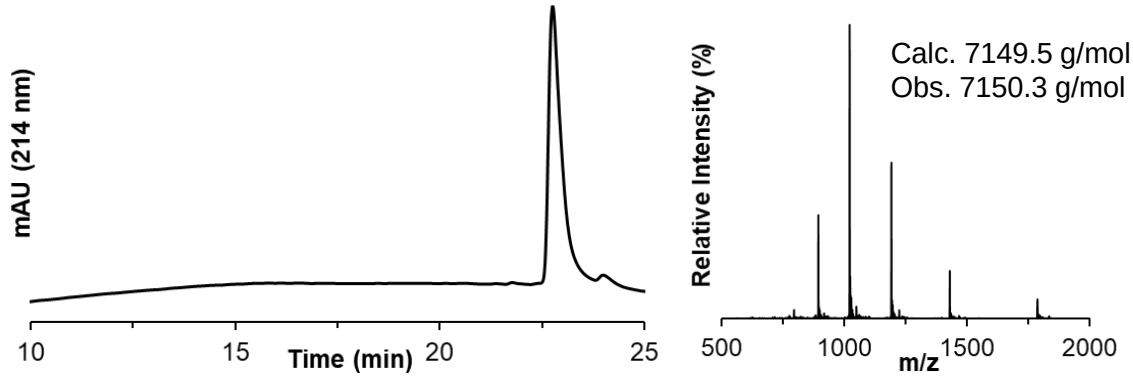


Figure S27. HPLC and LC/MS analysis of pure unfolded AS-48.

Section S16: CD Analysis of Folded AS-48 Protein

The purified synthetic AS-48 was dissolved (52 µM) in 700 µL of 6 M GuHCl and 100 mM sodium phosphate at pH 6.5. The protein was dialyzed in a single step using a slide-a-lyzer dialysis cassette (MWCO=3,500) into 100 mL of 20 mM sodium phosphate, pH 6.5 overnight at 4 ºC. After dialysis, the protein concentration was re-tested and remained at 52 µM. A small aliquot of the folded protein was diluted to 25 µM and analyzed by CD.

CD spectrum of synthetic AS-48 was obtained with an AVIV model 410 circular dichroism spectrometer. 20 mM sodium phosphate, pH 6.5 (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. Scans were then averaged and blank-subtracted before normalization to mean residue ellipticity ([θ] = 100 * θ/(C * l * n), where C is concentration of protein in mM, l is path length in centimeters, and n is the number of peptide bonds in the protein).

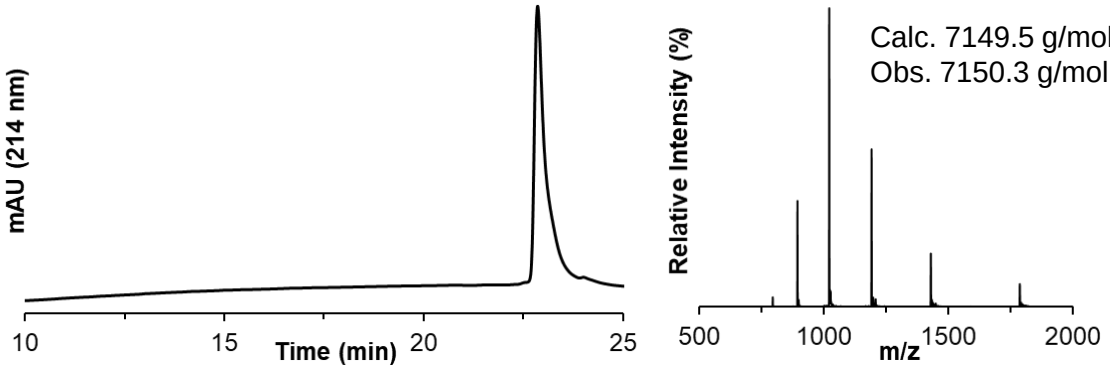


Figure S28. HPLC and LC/MS analysis of pure folded AS-48.

Section S17: Expression and Purification of Native AS-48

The Native AS-48 sample was obtained from cultures of *E. faecalis* UGRA10 producer strain[8] on Esprion 300 supplemented with 1% glucose as described previously.[9] AS-48 was collected from supernatants by cationic exchange chromatography on a carboxymethyl sepharose matrix, desalted, concentrated, and finally purified via RP-HPLC on a Vydac 218TP510 column. The concentration of the purified AS-48 sample was determined by measuring UV absorption at 280 nm.

Section S18: Activity Assays of Folded Synthetic AS-48

The range of antibacterial activity of native and synthetic AS-48 peptides (52 µM) was determined by the spot-on-lawn assay against four Gram-positive bacteria susceptible to AS-48 (*Listeria innocua* CECT 4030, *Enterococcus faecalis* S-47, *Staphylococcus aureus* CECT 240, and *Arthrobacter* sp) and two Gram-negative AS-48 resistant strains (*Escherichia coli* ATCC25922 and *Pseudomonas putida*).

Synthetic and native samples of AS-48 were dissolved in a 20 mM sodium phosphate buffer at pH 6.5 to 52 µM and serially two-fold diluted in sterile buffer. 3 µL spots of both samples were deposited onto plates of Mueller–Hinton agar (Scharlab) previously overlaid with 6 mL of buffered BHA or LB soft agar inoculated with the indicator Gram-positive and Gram-negative bacteria, respectively. Plates were incubated overnight at appropriate temperature (37 or 28ºC) and examined for halos of inhibition.

Once the activity of the synthetic AS-48 was confirmed, the minimal inhibitory concentration (MIC) of both protein samples were determined using the broth CLSI microdilution method.[10] MIC is defined as the concentration of AS-48 that prevents overnight growth as indicated by OD_{620}.

Serial two-fold dilutions of both authentic and synthetic AS-48 were used. Cultures of the susceptible bacteria grown for 24 h in appropriate media (BHA or LB agar) were adjusted with phosphate saline buffer to a turbidity equal to that of the 0.5 McFarland standard. The bacteria suspensions were further diluted to provide a final inoculum density of approximately 10^5 CFU/ml in each well of a 96-well plate. Diluted samples of AS-48 were added and incubated for 24–48 hours at 28 or 37° C. After

incubation, the MIC was read as the lowest concentration of AS-48 at which there was no visible growth. The absence of growth was also spectrophotometrically determined at 620 nm using a Tecan Spectrophotometer. Two independent experiments starting from two different protein stocks were performed.

Section S19: References

1. Y.-C. Huang , C.-C. Chen , S. Gao, Y.-H. Wang, H. Xiao, F. Wang, C.-L. Tian and Y.-M. Li, *Chemistry – A European Journal*, 2016, **22**, 7623-7628.
2. J. M. Fulcher, M. E. Petersen, R. J. Giesler, Z. S. Cruz, D. M. Eckert, J. N. Francis, E. M. Kawamoto, M. T. Jacobsen and M. S. Kay, *Org Biomol Chem*, 2019, **17**, 10237-10244.
3. M. T. Jacobsen, M. E. Petersen, X. Ye, M. Galibert, G. H. Lorimer, V. Aucagne and M. S. Kay, *J Am Chem Soc*, 2016, **138**, 11775-11782.
4. G. M. Fang, J. X. Wang and L. Liu, *Angew Chem Int Ed Engl*, 2012, **51**, 10347-10350.
5. P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. Kent, *Science*, 1994, **266**, 776-779.
6. Q. Wan and S. J. Danishefsky, *Angew Chem Int Ed Engl*, 2007, **46**, 9248-9252.
7. N. Kamo, G. Hayashi and A. Okamoto, *Chem Commun (Camb)*, 2018, **54**, 4337-4340.
8. R. Cebrian, A. Banos, E. Valdivia, R. Perez-Pulido, M. Martinez-Bueno and M. Maqueda, *Food Microbiol*, 2012, **30**, 59-67.
9. S. Ananou, A. Muñoz, A. Gálvez, M. Martínez-Bueno, M. Maqueda and E. Valdivia, *International Dairy Journal*, 2008, **18**, 923-927.
10. P. Wayne, *Performance standards for antimicrobial susceptibility testing*, 2010, **20**, 1-5.