Supplementary Information

Traceless Click-Assisted Native Chemical Ligation Enabled by Protecting Dibenzocyclooctyne from Acid-Mediated Rearrangement with Copper(I)

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Supplementary Information Contents

SECTION 1: Materials

Solid-Phase Peptide Synthesis (SPPS)

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-L-Asp(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, and Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH were purchased from Gyros Protein Technologies. Fmoc-L-Met-OH, Fmoc-L-Lys(Dde)-OH, and Fmoc-L-Norleucine-OH were purchased from AAPPTec. Boc-L-Cys(S*t*bu)-OH and 6-azidohexanoic acid (≥98%) were purchased from Chem-Impex International. Dibenzocyclooctyne-acid (DBCO-acid, 95%), acetic anhydride (99.5%), di-tert-butyl dicarbonate (ReagentPlus grade), anhydrous hydrazine (98%), *N,N*-diisopropylethylamine (DIPEA, ReagentPlus grade), Oxyma Pure (≥99%), *N,N'*-diisopropylcarbodiimide (DIC, 99%), 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]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, 99%) was purchased from Oakwood Chemical. Ninhydrin (ACS grade) was purchased from Acros Organics. 190 proof ethanol and 200 proof ethanol were purchased from Decon Laboratories. Fmoc-N-

amido-dPEG$_8$-acid was purchased from Quanta Biodesign. DBCO-C6-NHS ester (>95%) was purchased from Click Chemistry Tools. The traceless Lys linker *N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione (Fmoc-Ddap-OH) was synthesized as described in the established protocol.[1]

Peptide Cleavage and Precipitation

Trifluoroacetic acid (TFA, peptide synthesis grade) and anhydrous ethyl ether (ACS grade) were purchased from Fisher Scientific. Tetrakis(acetonitrile)copper(I) tetrafluoroborate ([MeCN]$_4$CuBF$_4$, 97%) and triisopropylsilane (TIS, 98%) were purchased from Sigma Aldrich. 1,2-ethanedithiol (EDT, 95%) and ammonium iodide (NH_4I, ≥99%) were purchased from Acros Organics. Thioanisole (≥99%) was purchased from Fluka.

RP-HPLC and LC/MS

Trifluoroacetic acid (TFA, HPLC grade) was purchased from Alfa Aesar. Acetonitrile (MeCN, 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.

Copper Quantitation

MQuant™ copper test strips were purchased from EMD Millipore. Nitric acid (ACS grade) was purchased from Acros Organics. Optima LC/MS water was purchased from Fisher Scientific.

Trypsin Digestion for High-resolution LC/MS

Ammonium bicarbonate and Pierce™ Trypsin Protease, MS Grade were purchased from Fisher Scientific.

Click, Ligation, Desulfurization, and Ddap Linker Cleavage

Guanidine hydrochloride (GnHCl, ≥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. Methyl thioglycolate (MTG, ≥98%) was purchased from TCI Chemicals. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl, ≥99%) was purchased from Hampton Research. VA-044 was purchased from Wako. L-glutathione reduced (≥98%) and hydroxylamine hydrochloride (ReagentPlus grade) were purchased from Sigma Aldrich.

SECTION 2: Methods

Automated Peptide Synthesis

All peptides were synthesized on a Prelude X instrument (Gyros Protein Technologies) using Fmoc SPPS. All deprotection and coupling cycles were performed with nitrogen bubbling, and gentle shaking was also used for couplings. All steps were performed at room temperature (rt) unless otherwise specified.

For 30 µmol scale syntheses, deprotection cycles consisted of three consecutive 3 min treatments of 2 mL 20% piperidine in DMF, followed by three 30 s washes of 2 mL DMF. Coupling cycles consisted of 25 min treatment with a solution 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, followed by three 30 s washes of 2 mL DMF.

For 100 µmol scale syntheses, deprotection cycles consisted of three consecutive 3 min treatments of 5 mL 20% piperidine in DMF, followed by three 30 s washes of 5 mL DMF. Coupling cycles consisted of 25 min treatment with a solution of 2.65 mL 200 mM amino acid in NMP, 2.65 mL 195 mM HATU in DMF, and 2 mL 600 mM NMM in DMF, followed by three 30 s washes of 5 mL DMF.

Washing, Swelling, and Mixing of Resins

For 30 µmol scale syntheses, resins were washed and swelled with approximately 3 mL of specified solvent. For mini scale manual syntheses, resins were washed and swelled with approximately 0.5 mL of specified solvent. The wash volumes for all other synthesis scales are indicated in the relevant sections below. All swelling and manual coupling/deprotection steps were mixed on a rotisserie at rt, unless otherwise specified.

Preparation of Peptide Resins

To synthesize C-terminal amide peptides at 30 μmol scale, 158 mg Tentagel R RAM resin (0.19 mmol/g) or 75 mg rink amide ChemMatrix resin (0.4 mmol/g) was weighed into a 6 mL SPPS tube. The resin was washed three times with DMF, followed by three DCM washes. The resin was then allowed to swell in DMF for ≥ 10 min, and the swelled resin was transferred to the Prelude X instrument for automated SPPS.

To synthesize C-terminal amide peptides at 100 μmol scale, 526 mg Tentagel R RAM resin (0.19 mmol/g) was weighed into a 6 mL SPPS tube. The resin was washed three times with ~5 mL DMF, followed by three washes of ~5 mL DCM. The resin was then allowed to swell in ~5 mL DMF for ≥ 10 min, and the swelled resin was transferred to the Prelude X instrument for automated SPPS.

To prepare C-terminal acid peptides at 30 μmol scale, 150 mg 2-chlorotrityl chloride resin (0.77 mmol/g) was weighed into a 6 mL SPPS tube. The resin was washed three times with DMF, followed by three DCM washes. In order to load the first amino acid onto the resin, 0.03 mmol of the Fmoc-protected amino acid was dissolved in 1 mL of a 1:1 DMF/DCM mixture. 0.15 mmol DIPEA (26 μL) was then mixed into the amino acid solution. The entire amino acid and DIPEA solution was added to the 2-chlorotrityl chloride resin, and the coupling reaction was rotated for 1 h. The resin was then washed three times with DCM. Unreacted 2-chlorotrityl chloride was capped by repeatedly washing the resin with a 17:2:1 DCM:MeOH:DIPEA mixture (individual washes with 15-30 s of shaking by hand, with ~20 total mL used). Once the capping reaction was complete, the resin was washed three times with DCM, followed by three

DMF washes. Finally, the resin was transferred to the Prelude X instrument for automated SPPS.

To synthesize C-terminal hydrazide peptides at 30 μmol scale, two methods were used:

- **Method A**: 2-chlorotrityl chloride resin was freshly converted into hydrazine resin based on an established protocol,[2] with several modifications. 150 mg 2-chlorotrityl chloride resin (0.77 mmol/g) was first weighed into a 6 mL SPPS tube. The resin was washed three times with DMF, followed by three DCM washes. The resin was then swelled in a 1:1 DMF/DCM mixture for ≥ 30 min. Hydrazine was generated on the resin through two separate 30 min treatments of 2 mL 5% hydrazine in DMF, with mixing. After each hydrazine treatment, the resin was washed three times with DMF. Unconverted resin was then capped using a 10 min treatment of 2 mL 5% MeOH in DMF, with mixing. After the capping treatment, the hydrazine resin was washed three times with DMF, followed by three DCM washes. The first amino acid was manually loaded onto the hydrazine resin by dissolving 0.03 mmol Fmoc-protected amino acid and 0.03 mmol Oxyma Pure in 1.2 mL of a 1:1 DMF/DCM mixture. 0.06 mmol DIC was added to the amino acid-Oxyma Pure solution, and the solution was pre-activated for 10 min on a rotisserie. The pre-activated solution was added to the hydrazine resin, and coupling occurred for 45 min. The resin was then washed six times with DMF. To cap any unreacted hydrazine on the resin, the resin was treated with 1 mL acetic anhydride mixed with 1 mL 600 mM NMM in DMF for 15 min on a rotisserie.

Finally, the resin was washed six times with DMF before being transferred to the Prelude X instrument for automated SPPS.

- **Method B**: Fmoc-hydrazine on 2-chlorotrityl chloride resin was prepared based on an established protocol,[3] with several modifications. 150 mg of 2-chlorotrityl chloride resin (0.77 mmol/g) was weighed into a 6 mL SPPS tube. The resin was washed three times with DCM. 1.2 mL DCM was then added to the resin, and the resin was allowed to swell for 10 min at 4°C. 30.6 μmol Fmoc-hydrazine (7.8 mg) was dissolved in a mixture of 1.5 mL DMF and 0.3 mL DCM, followed by addition of 266 μL DIPEA. Once the 2-chlorotrityl chloride resin was finished swelling, the Fmoc-hydrazine and DIPEA solution was added to the resin slurry at 4°C. The resin slurry was then placed on a rotisserie at rt, and the coupling reaction was rotated for 2 h. Once the coupling reaction was finished, 30 μL MeOH was added to the resin (without draining the SPPS tube) in order to cap unreacted 2-chlorotrityl chloride (rotated for 10 min). The resin was then washed three times with DMF, followed by three DCM washes. The resin was dried under vacuum for ≥ 2 min before storage in a 4°C desiccator. Prior to use, the resin was warmed to rt before being washed three times with DMF, followed by three DCM washes. The resin was then swelled in a 1:1 DMF/DCM mixture for ≥ 10 min. All of the swelled resin was then transferred to the Prelude X instrument for automated SPPS. This procedure generates resin with a loading density similar to Tentagel R RAM resin (approximately 0.2 mmol/g).

To synthesize C-terminal hydrazide peptides at 60 μmol scale, Fmoc-hydrazine on 2-chlorotrityl chloride resin was prepared as described in an established protocol.[3] The loading density of this resin was determined to be 0.8 mmol/g. 75 mg resin was placed into a 6 mL SPPS tube. The resin was washed three times with ~3 mL DMF, followed by three washes of ~3 mL DCM. The resin was then swelled in ~3 mL of a 1:1 DMF/DCM mixture for ≥ 10 min. The swelled resin was then transferred to the Prelude X for SPPS (same coupling and deprotecting cycles as the 30 μmol scale synthesis methods).

To synthesize C-terminal hydrazide peptides at 100 μmol scale, 2-chlorotrityl chloride resin was freshly converted into hydrazine resin based on an established protocol,[2] with several modifications. ~500 mg 2-chlorotrityl chloride resin (0.77 mmol/g) was placed into a 20 mL SPPS tube. The resin was washed three times with ~10 mL DMF, followed by three washes of ~10 mL DCM. The resin was then swelled in ~10 mL of a 1:1 DMF/DCM mixture for ≥ 30 min. Hydrazine was generated on the resin through two separate 30 min treatments of 8 mL 5% hydrazine in DMF, with mixing. After each hydrazine treatment, the resin was washed three times with ~10 mL DMF. Unconverted resin was capped with a 10 min treatment of 8 mL 5% MeOH in DMF, with mixing. After the capping treatment, the hydrazine resin was washed three times with ~10 mL DMF, followed by three washes of ~10 mL DCM. The first amino acid was manually loaded onto the hydrazine resin by first dissolving 0.1 mmol Fmoc-protected amino acid and 0.1 mmol Oxyma Pure in 4 mL of a 1:1 DMF/DCM mixture. 0.2 mmol DIC was added, and the solution was pre-activated for 10 min on a rotisserie. The pre-activated solution was then added to the hydrazine resin and coupled for 45 min. The resin was then washed

six times with ~10 mL DMF. To cap any unreacted hydrazine, the resin was treated with 3.3 mL acetic anhydride mixed with 3.3 mL 600 mM NMM in DMF for 15 min on a rotisserie. Finally, the resin was washed six times with ~10 mL DMF before being transferred to the Prelude X for SPPS.

N-terminal Capping Procedures

N-terminal peptide acetylation at 30 µmol scale was achieved through 20 min treatment of 1 mL acetic anhydride combined with 1 mL 600 mM NMM in DMF, with mixing. The resin was then washed three times with DMF, followed by three DCM washes.

N-terminal peptide acetylation at 100 µmol scale was achieved through 20 min treatment of 4 mL acetic anhydride mixed with 4 mL 600 mM NMM in DMF, with gentle shaking of the peptide resin on the Prelude X instrument. The resin was then washed three times with ~3 mL DMF, followed by three washes of ~3 mL DCM.

N-terminal Boc protection at 30 µmol scale was completed through two consecutive 20 min treatments of 0.7 mL 200 mM di-tert-butyl dicarbonate in DMF mixed with 0.5 mL 600 mM NMM in DMF, with mixing. Following the Boc treatments, the resin was washed three times with DMF, followed by three DCM washes.

Manual Peptide Synthesis

In order to couple various modifications on specific Lys residues (e.g., Ddap linker, DBCO, or azide), manual couplings/deprotections were performed on peptides containing a single Lys(Dde) residue after automated peptide synthesis and N-terminal capping was completed. Manual peptide syntheses were generally completed at either 30 µmol scale (to prepare enough peptide for purification) or mini scale (to develop

various synthesis and cleavage methods). For mini scale syntheses, peptide resins were prepared through one of two methods:

- **Mini Scale Method A**: After automated peptide synthesis and N-terminal capping was complete, 100 μmol scale or 30 μmol scale peptide resin was washed three times with DMF, followed by three washes of DCM. The resin was allowed to dry under vacuum for ≥ 30 min, and then specific amounts of peptide resin were weighed into 1 mL SPPS tubes. For peptides prepared on Tentagel R RAM resin or 2-chlorotrityl chloride resin, ~15 mg of dry resin was weighed into the SPPS tube. For peptides prepared on rink amide ChemMatrix resin, ~7.5 mg of dry resin was weighed into the SPPS tube (the loading density of this resin was ~2x higher than Tentagel R RAM and 2-chlorotrityl chloride resins). Peptide resins were then swelled in DMF for ≥ 30 min before mini scale syntheses were started.
- **Mini Scale Method B:** After automated peptide synthesis and N-terminal capping was complete, 30 μmol scale peptide resin was evenly distributed into 10 separate 1 mL SPPS tubes. Distribution was performed by suspending the resin in a set volume of DMF, placing 1/10 of the total volume into each 1 mL SPPS tube, and then removing the DMF from each SPPS tube. If all tubes did not contain approximately the same amount of resin, DMF was added to tubes with more resin, the resin was resuspended, and a small amount of resin was redistributed until all 10 tubes contained approximately the same amount of resin (3 μmol). Peptide resins could then be swelled in DMF for ≥ 30 min to prepare for mini scale syntheses.

Alternatively, the resins could be washed three times with DMF, followed by three DCM washes, dried under vacuum for ≥ 30 min, and stored in a 4°C desiccator.

Kaiser Test

To monitor the progress of manual deprotections and couplings, the Kaiser test[4] was used to observe the presence of primary amines on peptide resin. 6% ninhydrin in ethanol (either 190 or 200 proof) was used as the Kaiser reagent. To complete the Kaiser test, a few beads of peptide resin were aliquoted into a 1.5 mL Eppendorf tube. If the resin was not washed with DMF beforehand, the resin aliquot was washed with 300 µL ethanol (either 190 or 200 proof). The resin aliquot was then mixed with 50 µL Kaiser reagent and vortexed for 3 s. The mixture was spun down for 3 s on a tabletop centrifuge and incubated at 90°C for 5 min. If the solution and/or beads were blue/purple, then the resin was assumed to contain unreacted primary amines. If the Kaiser test gave an unexpected result, another round of deprotection or coupling was performed.

Manual Lys(Dde) Deprotection

For 30 µmol scale Lys(Dde) deprotection, peptide resin was treated once with 2.5 mL 5% hydrazine in DMF for 20 min, with mixing. Following the hydrazine treatment, the resin was washed six times with DMF.

For mini scale Lys(Dde) deprotection, peptide resin was treated once with 500 µL 5% hydrazine in DMF for 10 min, with mixing. Following the hydrazine treatment, the resin was washed three times with DMF.

Manual Fmoc-Ddap-OH Coupling

Coupling of the helping hand Lys linker (Fmoc-Ddap-OH) was completed based on the established protocol[1] at 30 µmol scale. Peptide resin containing a single primary amine on a specific Lys side chain was treated once with 1 mL 200 mM Fmoc-Ddap-OH in NMP overnight (15-20 h), with mixing on a rotisserie at 37°C. After the coupling reaction was complete, the peptide resin was washed three times with DMF. Note that the unreacted Fmoc-Ddap-OH in NMP can be stored in -20°C for recycling.

Manual Fmoc Deprotection

For 30 µmol scale syntheses, Fmoc was manually deprotected through three consecutive 3 min treatments of 2 mL 20% piperidine in DMF, with mixing. After the third treatment, the resin was washed three times with DMF.

Manual Fmoc-Lys(Boc)-OH Coupling

For 30 µmol scale syntheses, Fmoc-Lys(Boc)-OH was manually coupled by adding 700 µL 200 mM Fmoc-Lys(Boc)-OH in NMP, 700 µL 195 mM HATU in DMF, and 500 µL 600 mM NMM in DMF to the peptide resin. The coupling reaction was rotated for 25 min. The resin was then washed three times with DMF.

Manual Fmoc-N-amido-dPEG$_8$-acid Coupling

Manual coupling of PEG_8 at 30 µmol scale was achieved by adding 1.5 mL 42 mM Fmoc-N-amido-dPEG$_8$-acid in DMF, 0.3 mL 195 mM HATU in DMF, and 0.3 mL 600 mM NMM in DMF to the peptide resin. The coupling reaction was rotated for 1 h. The peptide resin was then washed three times with DMF.

Manual 6-azidohexanoic Acid Coupling

Coupling of 6-azidohexanoic acid to 30 μmol scale peptide on resin was achieved through a single 1 h treatment of 0.7 mL 200 mM 6-azidohexanoic acid in DMF, 0.7 mL 195 mM HATU in DMF, and 0.5 mL 600 mM NMM in DMF, with mixing. Resin was washed six times with DMF, followed by three DCM washes.

For mini scale syntheses, 6-azidohexanoic acid coupling was achieved through a single 1 h treatment of 0.2 mL 200 mM 6-azidohexanoic acid in NMP, 0.2 mL 195 mM HATU in DMF, and 0.143 mL 600 mM NMM in DMF, with mixing. Resin was washed three times with DMF, followed by three DCM washes.

Manual DBCO-C6-NHS Ester Coupling

Coupling of DBCO to 30 μmol scale peptide on resin was generally achieved through one 2 h treatment of 1 mL 45 mM DBCO-C6-NHS ester in DMF mixed with 1 mL 300 mM DIPEA in DMF, with mixing of the peptide resin on rotisserie at 37°C. When coupling DBCO to N-terminal amines, additional steps were required for complete coupling. For peptide **6b** (see **Table S1**), an additional 2 h of coupling at 37°C was required. For peptide **13b** (see **Table S1**), an additional 2 h of coupling at 37°C, followed by a second overnight coupling at 50°C with fresh DBCO-C6-NHS ester and DIPEA solutions, was required. Once the coupling reaction was complete, the resin was washed six times with DMF, followed by three DCM washes.

For mini scale peptide syntheses, DBCO was generally coupled through 1 h treatment of 0.1 mL 33 mM DBCO-C6-NHS ester in DMF mixed with 0.1 mL 300 mM DIPEA in DMF, with mixing. Resin was then washed three times with DMF, followed by three DCM washes.

On-resin Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC)

On-resin SPAAC reactions were performed on mini scale peptide syntheses following DBCO-C6-NHS ester coupling. DBCO peptide resins were swelled in DMF for ≥ 10 min. The resins were then treated for 1 h with 0.2 mL 75 mM 6-azidohexanoic acid in DMF, with mixing. After SPAAC, the resin was washed three times with DMF, followed by three DCM washes.

On-resin Protection of DBCO with $(MeCN)_4CuBF_4$ Dissolved in Organic Solvent

After DBCO coupling, mini scale peptide resins were treated for 1 h with 0.2 mL 30 mM $(MeCN)_4CuBF_4$ in organic solvent (DMF, MeOH, DCM, NMP, or MeCN), with mixing. The resin was then washed six times with the same solvent used to dissolve $(MeCN)_4CuBF_4$, followed by three DMF washes, and finally three DCM washes. The resins were then dried under vacuum for ≥ 30 min in preparation for resin cleavage and precipitation.

Peptide Resin Storage and Handling

Once all desired deprotections and couplings were complete, peptide resin was washed ≥ 3 times with DMF, followed by three washes with DCM. The resin was dried under vacuum for ≥ 30 min before being stored in a 4°C desiccator. If additional deprotections and couplings needed to be performed on dried resin, it was equilibrated to rt and swelled in DMF for ≥ 30 min.

Cleavage and Peptide Precipitation

30 µmol scale peptide cleavage was typically accomplished with a 3 h treatment of 4 mL TFA containing 2.5% water and 2.5% TIS, with mixing on a rotisserie at rt. For peptides containing Met, 2.5% EDT and ~100 mg NH_4I were added to the TFA cocktail,

unless DBCO or azide was also in the peptide. For peptides containing DBCO, 5 equiv $(MeCN)_4CuBF_4$ (150 μmol, or 47.2 mg) was added to the dried peptide resin before addition of the standard TFA cocktail (95% TFA, 2.5% water, and 2.5% TIS). After cleavage, the TFA solution was added to ~40 mL ice-cold ethyl ether, shaken thoroughly, and placed at -20°C for ≥ 30 min, in order to precipitate the crude peptide. The solution was then centrifuged at 4,700 *g*, 4°C for ≥ 10 min. The resulting supernatant was decanted, and crude peptide pellets were washed twice with ~40 mL ice-cold ethyl ether. The crude peptide pellets were dried in a vacuum desiccator overnight.

Mini scale peptide cleavage was generally performed with a 3 h treatment of 500 μL TFA containing 2.5% water and 2.5% TIS, with mixing on a rotisserie. For some peptide resins, 2.5% EDT, 2.5% thioanisole, or ~10 mg NH_4I were added to the TFA cocktail, as indicated in relevant figure legends. To protect DBCO peptides during cleavage, typically 1-5 mg $(MeCN)_4CuBF_4$ was added to the dried peptide resin before addition of the standard TFA cocktail (see below method for calculating $(MeCN)_4CuBF_4$ equiv used for mini scale syntheses). After cleavage, the TFA solution was added to ~5-8 mL ice-cold ethyl ether, shaken thoroughly, and placed in -20°C for ≥ 30 min, in order to precipitate the crude peptide. If precipitate was not observed, ethyl ether was slowly evaporated until precipitate was seen. The solution was then centrifuged at 4,700 *g*, 4°C for 20 min. The resulting supernatant was decanted, and the crude peptide pellets were washed two times with ~7-8 mL ice-cold ethyl ether. The crude peptide pellets were finally dried in a vacuum desiccator for ≥ 1 h.

Notably, we found that including 2.5% thioanisole or 2.5% EDT diminished the protective properties of the Cu(I) additive (peptide **3a**, data not shown). Furthermore, we noted that NH_4I, a cleavage additive used to reverse Met oxidation,[5, 6] was also capable of completely removing the S*t*Bu-protecting group (peptides **3a** and **3b**, data not shown).

Calculating $(MeCN)_4CuBF_4$ Equiv for DBCO Protection in Mini Scale Syntheses

Depending on the mini scale peptide synthesis method used (see the "Manual Peptide Synthesis" methods section), calculating the $(MeCN)_4CuBF_4$ equiv for DBCO protection experiments was done through one of two techniques:

- **If Mini Scale Method A was used** (weighing peptide resin into SPPS tubes): The number of moles of peptide resin was calculated based on the weight of peptide resin used for manual couplings and the expected weight of peptide resin after automated peptide synthesis, assuming the entire 30 or 100 μmol peptide was generated. Dividing the moles of $(MeCN)_4CuBF_4$ used in cleavage by the calculated moles of peptide resin gave the $(MeCN)_4CuBF_4$ equiv used.
- **If Mini Scale Method B was used** (splitting 30 μmol scale peptide equally into 10 SPPS tubes): The number of moles of peptide resin was estimated to be 3 μmol, as the 30 μmol scale peptide resin was equally split into 10 tubes. As a result, the $(MeCN)_4CuBF_4$ equiv was simply calculated by dividing the moles of $(MeCN)_4CuBF_4$ used by 3 μmol.

Due to the simplicity and accuracy of determining $(MeCN)_4CuBF_4$ equiv with mini scale peptide synthesis method B, this method was used when the $(MeCN)_4CuBF_4$

equiv used in cleavages needed to be carefully measured (e.g., experiments for determining the optimal amount of $(MeCN)_4CuBF_4$ to add during peptide cleavage; see **Figures 3(C)** and **S15-17**).

Preparation of Crude Peptides for HPLC Analyses

30 μmol scale crude peptides were dissolved in 9-18% MeCN 0.1% TFA (~20-40 mL total volume) and were vortexed and sonicated to dissolve as much material as possible, followed by centrifugation at 4,700 *g*, 4°C for 10 min. The supernatants were then filtered through a 0.22 μm filter prior to HPLC or LC/MS.

Mini scale crude peptides were generally dissolved in 18% MeCN 0.1% TFA (~1-5 mL) and were then vortexed and sonicated to dissolve as much material as possible. If the sample volume was ~1 mL, the sample was centrifuged at 18,000 *g*, rt for 10 min prior to HPLC and/or LC/MS. If the sample volume was ~5 mL, the sample was centrifuged at 4,700 *g*, 4°C for 10 min prior to HPLC and/or LC/MS.

Note that crude DBCO peptides protected with $(MeCN)_4CuBF_4$ during cleavage have darker pellets after precipitation. They also usually have a small amount of dark-colored material that does not dissolve in aqueous buffer, presumably derived from oxidized copper species. Therefore, centrifugation prior to HPLC or LC/MS is critical.

Analytical LC/MS Methods

0.1% formic acid in water (Buffer A) and 0.1% formic acid in MeCN (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). Chromatograms were corrected based on

the baseline absorbance detected at 360 nm, unless specified in the figure legends. Unless otherwise noted, observed masses were manually calculated using the charge states from averaged scans across the major ion signal and corresponding UV peak (horizontal lines underneath chromatograms). Note that the deconvoluted mass spectra in **Figures 2, 5,** and **9** were generated using the UniDec software.[7] Calculated and observed masses are presented as average mass. Specific LC/MS methods are described in more detail below:

- **LC/MS Method A:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-1 min 5% B, 1-8 min 5-90% B, 8-8.1 min 90-5% B, 8.1-10 min 5% B; flow rate: 0-1 min 0.5 mL/min, 1-8 min 0.5-1.0 mL/min, 8-10 min 1.0 mL/min; scan range: 400-2,000 *m/z*; voltage: 90 V
- **LC/MS Method B:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-1 min 5% B, 1-1.1 min 5-10% B, 1.1-10 min 10-90% B, 10-11 min 90% B, 11-11.1 min 90-5% B, 11.1-13 min 5% B; flow rate: 0-1.1 min 0.5 mL/min, 1.1-10 min 0.5-1.0 mL/min, 10-13 min 1.0 mL/min; scan range: 50-1,000 *m/z*; voltage: 120 V
- **LC/MS Method C:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-1 min 5% B, 1-8 min 5-90% B, 8-8.1 min 90-5% B, 8.1-10 min 5% B; flow rate: 0-8 min 0.75 mL/min, 8-8.1 min 0.75-1.0 mL/min, 8.1-10 min 1.0 mL/min; scan range: 400-2,000 *m/z*; voltage: 90 V
- **LC/MS Method D:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-1 min 20% B, 1-11 min 20-50% B, 11-11.1 min 50-90% B,

11.1-12 min 90% B, 12-12.1 min 90-20% B, 12.1-14 min 20% B; flow rate: 0-14 min 1.0 mL/min; scan range: 400-2,000 *m/z*; voltage 90 V

- **LC/MS Method E:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 45°C; gradient: 0-5 min 5% B, 5-35 min 5-40% B; 35-35.1 min 40-90% B, 35.1-36 min 90% B, 36-36.1 min 90-5% B, 36.1-38 min 5% B; flow rate: 0-38 min 1.0 mL/min; scan range: 400-2,000 *m/z*; voltage 90 V
- **LC/MS Method F:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-5 min 10% B, 5-35 min 10-50% B, 35-35.1 min 50-90% B, 35.1-36 min 90% B, 36-36.1 min 90-10% B, 36.1-40 min 10% B; flow rate: 0-40 min 1.0 mL/min; scan range: 400-2,000 *m/z*; voltage 90 V
- **LC/MS Method G:** Agilent Poroshell 2.7 µm EC-C18 (120 Å, 4.6 x 50 mm); 50°C; gradient: 0-1 min 5% B, 1-8 min 5-90% B, 8-8.1 min 90-5% B, 8.1-10 min 5% B; flow rate: 0-8 min 0.75 mL/min, 8-8.1 min 0.75-1.0 mL/min, 8.1-10 min 1.0 mL/min; scan range: 200-1,200 *m/z*; voltage: 50 V

Analytical HPLC Methods

0.1% TFA in water (Buffer A) and 0.1% TFA in 90% MeCN (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:

- **Analytical Method A:** Phenomenex Luna 5 µm C18(2) (100 Å, 4.6 x 250 mm); 45°C; gradient: 0-5 min 10% B, 5-35 min 10-70% B, 35-35.1 min 70-90% B, 35.1-36 min 90% B, 36-36.1 min 90-10% B, 36.1-40 min 10% B; flow rate: 2.0 mL/min

- **Analytical Method B:** Phenomenex bioZen 3.6 µm Intact C4 (200 Å, 4.6 x 150 mm); 45°C; gradient: 0-2 min 6% B, 2-12.5 min 6-31.5% B, 12.5-12.6 min 31.5-90% B, 12.6-13.5 min 90% B, 13.5-14.5 min 90-6% B, 14.5-18.5 min 6% B; flow rate: 2.0 mL/min
- **Analytical Method C:** Phenomenex bioZen 3.6 µm Intact C4 (200 Å, 4.6 x 150 mm); 45°C; gradient: 0-5 min 10% B, 5-20 min 10-50% B, 20-20.1 min 50-90% B, 20.1-22 min 90% B, 22-22.1 min 90-10% B, 22.1-25 min 10% B; flow rate: 2.0 mL/min
- **Analytical Method D:** Phenomenex Aeris 3.6 µm WIDEPORE C4 (200 Å, 4.6 x 150 mm); 40°C; gradient: 0-2 min 10% B, 2-32 min 10-55% B, 32-32.1 min 55-100% B, 32.1-34 min 100% B, 34-34.1 min 100-10% B, 34.1-40 min 10% B; flow rate: 1.0 mL/min
- **Analytical Method E:** Phenomenex Kinetex 5 µm C18 (100 Å, 4.6 x 150 mm); 40°C; gradient: 0-2 min 10% B, 2-32 min 10-55% B, 32-32.1 min 55-100% B, 32.1-34 min 100% B, 34-34.1 min 100-10% B, 34.1-40 min 10% B; flow rate: 1.0 mL/min

Preparative HPLC Methods

0.1% TFA in water (Buffer A) and 0.1% TFA in 90% MeCN (Buffer B) were used as mobile phases for preparative HPLC purifications. Peptide purifications were performed on an Agilent 1260 Infinity II LC system, Agilent 1260 Infinity II preparative system, or Beckman Gold 126 HPLC. Specific purification methods are described below:

- **Purification Method A:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 10% B, 4-5 min 10-30% B, 5-30 min 30-60% B, 30-31 min 60-90% B, 31-36 min 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 4.0 mL/min
- **Purification Method B:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 10% B, 4-5 min 10-40% B, 5-30 min 40-55% B, 30-31 min 55-90% B, 31-36 min 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 4.0 mL/min
- **Purification Method C:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 10% B, 4-5 min 10-42% B, 5-30 min 42-48% B, 30-31 min 48-90% B, 31-36 min 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 4.0 mL/min
- **Purification Method D:** Waters Xbridge Peptide BEH300 10 µm C18 (300 Å, 19 x 250 mm); rt; gradient: 0-6 min 10% B, 6-31 min 10-50% B, 31-31.5 min 50-100% B, 31.5-32.5 min 100% B, 32.5-33 min 100-10% B, 33-38 min 10% B; flow rate: 10.0 mL/min
- **Purification Method E:** Waters Xbridge Peptide BEH300 10 µm C18 (300 Å, 19 x 250 mm); rt; gradient: 0-4 min 20% B, 4-19 min 20-50% B, 19-19.1 min 50-100% B, 19.1-21 min 100% B, 21-21.1 min 100-20% B, 21.1-25 min 20% B; flow rate: 20.0 mL/min
- **Purification Method F:** Waters Xbridge Peptide BEH300 10 µm C18 (300 Å, 19 x 250 mm); rt; gradient: 0-4.1 min 20% B, 4.1-19 min 20-50% B, 19-19.1 min 50-100% B, 19.1-21 min 100% B, 21-21.1 min 100-20% B, 21.1-25 min

20% B; flow rate: 0-4 min 10.0 mL/min, 4-4.1 min 10.0-20.0 mL/min, 4.1-25 min 20.0 mL/min

- **Purification Method G:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-10 min 10% B, 10-11 min 10-40% B, 11-36 min 40-55% B, 36-37 min 55-90% B, 37-42 min 90% B, 42-43 min 90-10% B, 43-46 min 10% B; flow rate: 4.0 mL/min
- **Purification Method H:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-5 min 10% B, 5-30 min 10-50% B, 30-31 min 50-90% B, 31-35 min 90% B, 35-35.1 min 90-10% B, 35.1-40 min 10% B; flow rate: 4.0 mL/min
- **Purification Method I:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 10% B, 4-5 min 10-43% B, 5-30 min 43-58% B, 30-31 min 58-90% B, 31-36 min 90% B, 36-37 min 90-10% B, 37-40 min 10% B; flow rate: 4.0 mL/min
- **Purification Method J:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-10 min 10% B, 10-11 min 10-43% B, 11-36 min 43-58% B, 36-37 min 58-90% B, 37-42 min 90% B, 42-43 min 90-10% B, 43-46 min 10% B; flow rate: 4.0 mL/min
- **Purification Method K:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5.1 min 10% B, 5.1-30 min 10-50% B, 30-30.1 min 50-100% B, 30.1-32 min 100% B, 32-32.1 min 100-10% B, 32.1-40 min 10% B; flow rate: 9.5 mL/min

- **Purification Method L:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 10% B, 5-5.1 min 10-15% B, 5.1-30 min 15-35% B, 30-30.1 min 35-100% B, 30.1-32 min 100% B, 32-32.1 min 100-10% B, 32.1-40 min 10% B; flow rate: 9.5 mL/min
- **Purification Method M:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 10% B, 5-5.1 min 10-18% B, 5.1-30 min 18-28% B, 30-30.1 min 28-100% B, 30.1-32 min 100% B, 32-32.1 min 100-10% B, 32.1-40 min 10% B; flow rate: 9.5 mL/min
- **Purification Method N:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 10% B, 5-5.1 min 10-19% B, 5.1-30 min 19-26% B, 30-30.1 min 26-100% B, 30.1-32 min 100% B, 32-32.1 min 100-10% B, 32.1-40 min 10% B; flow rate: 9.5 mL/min
- **Purification Method O:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 10% B, 5-5.1 min 10-18% B, 5.1-30 min 18-26% B, 30-30.1 min 26-100% B, 30.1-32 min 100% B, 32-32.1 min 100-10% B, 32.1-40 min 10% B; flow rate: 9.5 mL/min
- **Purification Method P:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-6 min 10% B, 6-31 min 10-30% B, 31-32 min 30-100% B, 32-36 min 100% B, 36-41 min 100-10% B, 41-46 min 10% B; flow rate: 10.0 mL/min
- **Purification Method Q:** Waters Xbridge Peptide BEH300 10 µm C18 (300 Å, 19 x 250 mm); rt; gradient: 0-6 min 20% B, 6-31 min 20-60% B, 31-32 min 60-100% B, 32-36 min 100% B, 36-41 min 100-20% B, 41-46 min 20% B; flow rate: 10.0 mL/min

- **Purification Method R:** Waters Xbridge Peptide BEH300 10 µm C18 (300 Å, 19 x 250 mm); rt; gradient: 0-6 min 15% B, 6-31 min 15-35% B, 31-32 min 35-100% B, 32-36 min 100% B, 36-41 min 100-15% B, 41-46 min 15% B; flow rate: 10.0 mL/min
- **Purification Method S:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-2 min 10% B, 2-2.1 min 10-15% B, 2.1-22 min 15-30% B, 22-22.1 min 30% B, 22.1-23 min 30-35% B, 23-23.1 min 35-10% B, 23.1-25 min 10% B; flow rate: 20.0 mL/min
- **Purification Method T:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5.1 min 18% B, 5.1-20 min 18-26% B, 20-20.1 min 26-90% B, 20.1-21 min 90-20% B, 21-24 min 20% B; flow rate: 0-5 min 10.0 mL/min, 5-5.1 min 10.0-30.0 mL/min, 5.1-20 min 30.0 mL/min, 20-20.1 min 30.0-20.0 mL/min, 20.1-22 min 20.0 mL/min, 22-24 min 20.0-0.0 mL/min
- **Purification Method U:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 20% B, 5-5.1 min 20-22% B, 5.1-20 min 22-30% B, 20-20.1 min 30-90% B, 20.1-21 min 90% B, 21-21.1 min 90-20% B, 21.1-24 min 20% B; flow rate: 0-5 min 10.0 mL/min, 5-5.1 min 10.0-20.0 mL/min, 5.1-24 min 20.0 mL/min
- **Purification Method V:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 20% B, 4-5 min 20-25% B, 5-30 min 25-40% B, 30-31 min 40-90% B, 31-36 min 90% B, 36-37 min 90-20% B, 37-40 min 20% B; flow rate: 4.0 mL/min

- **Purification Method W:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-4 min 20% B, 4-5 min 20-32% B, 5-30 min 32-40% B, 30-31 min 40-90% B, 31-36 min 90% B, 36-37 min 90-20% B, 37-40 min 20% B; flow rate: 4.0 mL/min
- **Purification Method X:** Phenomenex Kinetex 5 µm C18 (100 Å, 10 x 250 mm); 45°C; gradient: 0-10 min 20% B, 10-11 min 20-32% B, 11-36 min 32-40% B, 36-37 min 40-90% B, 37-42 min 90% B, 42-43 min 90-20% B, 43-46 min 20% B; flow rate: 4.0 mL/min
- **Purification Method Y:** Welch Ultisil 5 µm XB-C4 (120 Å, 10 x 150 mm); 50°C; gradient: 0-4 min 10% B, 4-5 min 10-20% B, 5-40 min 20-45% B, 40-45 min 45-90% B; flow rate: 9.0 mL/min
- **Purification Method Z:** Welch Ultisil 5 µm XB-C4 (120 Å, 21.2 x 150 mm); rt; gradient: 0-5 min 20% B, 5-5.1 min 20-22% B, 5.1-20 min 22-35% B, 20-20.1 min 35-90% B, 20.1-21 min 90% B, 21-21.1 min 90-20% B, 21.1-24 min 20% B; flow rate: 0-5 min 10.0 mL/min, 5-5.1 min 10.0-20.0 mL/min, 5.1-24 min 20.0 mL/min

Fractions collected during preparative HPLC purifications were analyzed by LC/MS in order to assess purity. All pure fractions were then pooled and analyzed by analytical HPLC and/or LC/MS. Finally, the pooled material was lyophilized to obtain dry, pure peptide (stored in a 4°C desiccator).

Note that during purification of DBCO peptides, the crude peptide solution and collected fractions were stored on ice in order to reduce DBCO degradation from the 0.1% TFA in HPLC buffers.

Preparation of HPLC and LC-MS Traces

HPLC and LC-MS traces were prepared for publication using our in-house Automated Trace Maker (ATM) programs. Using .CSV files of HPLC or LC-MS data, these Python scripts generate the desired chromatograms that can be viewed in Microsoft Excel. The ATM programs are available for free use on the Kay Lab Github website: https://github.com/kay-lab.

Calculation of DBCO A_{280} and A_{308} Extinction Coefficients

A 6.0 mM solution of Dibenzocyclooctyne-acid (DBCO-acid) was prepared by dissolving 1.0 mg DBCO-acid in 500 µL HPLC Buffer B (0.1% TFA in 90% MeCN). This 6.0 mM DBCO-acid solution was used in a 2-fold dilution series down to 11.71875 µM. For each dilution, triplicate absorbance measurements were taken at 280 nm and 308 nm using a NanoDrop One^C instrument (Thermo Scientific) blanked with HPLC Buffer B. The average A_{280} and A_{308} values were then calculated for each concentration. Since the average absorbance values were less than 0.5 for the two least concentrated samples (23.4375 µM and 11.71875 µM), these two concentrations were not used to calculate the extinction coefficients. For each of the remaining concentrations, extinction coefficients at 280 nm and 308 nm were calculated using Beer's Law. Finally, the average extinction coefficient was calculated for each wavelength.

The DBCO extinction coefficients were estimated as:

- 280 nm – ~14,000 $M^{-1}cm^{-1}$
- 308 nm – ~15,800 $M^{-1}cm^{-1}$

Calculation of Peptide Concentrations

For peptides containing Trp, Tyr, S*t*Bu-protected Cys, Ddap, and/or DBCO, in-solution concentrations were determined using A_{280} measurements collected on a NanoDrop One^{C} instrument. The following extinction coefficients were used to calculate concentrations via Beer's Law:

- Trp – 5,500 $M^{-1}cm^{-1}$
- Tyr – 1,490 $M^{-1}cm^{-1}$
- S*t*Bu-Cys – 125 $M^{-1}cm^{-1}$
- Ddap – 14,600 $M^{-1}cm^{-1}$
- DBCO – 14,000 $M^{-1}cm^{-1}$

Note that the A_{308} can be used to determine concentrations of DBCO peptides that do not contain the Ddap linker. For peptides without groups that absorb at 280 nm, concentrations were estimated by peptide weight. Alternatively, if the peptide was dissolved in a compatible buffer, the concentration was measured with a Direct Detect Infrared Spectrometer (Millipore Sigma). Note that peptide solubilizing buffers (e.g., 6 M GnHCl) are not compatible with the Direct Detect system.

SPAAC between 6-azidohexanoic Acid and DBCO Peptides in Aqueous Buffers

To determine if crude DBCO peptides could successfully undergo SPAAC, they were typically treated with 6-azidohexanoic acid shortly after being dissolved in 18% MeCN 0.1% TFA for HPLC and LC/MS analyses. Dissolved peptides were stored at 4°C if SPAAC could not be performed the same day (SPAAC was performed within a few days to avoid potential DBCO degradation). For the SPAAC reaction, a 62 mM 6-azidohexanoic acid in 18% MeCN 0.1% TFA buffer was prepared. 190 µL of crude

DBCO peptide solution was then mixed with 10 µL 62 mM 6-azidohexanoic acid, and the SPAAC reaction was placed on a rotisserie at 37°C for 6.5-26 h. SPAAC reactions were then centrifuged at 18,000 *g*, rt for 10 min before LC/MS analysis.

In order to perform SPAAC on a purified DBCO peptide containing a C-terminal hydrazide (peptide **1c** in **Table S1**; see **Figure S10**), the pure, lyophilized peptide was dissolved in 18% MeCN 0.1% TFA. Peptide concentration was determined using A_{308} as described above (extinction coefficient = 15,800 $M^{-1}cm^{-1}$). SPAAC was initiated by preparing 200 µL of 124 µM DBCO peptide, 231 µM 6-azidohexanoic acid in 18% MeCN 0.1% TFA. The reaction was placed on a rotisserie at 37°C for 2 h, and time points were taken at 0, 1, and 2 h. Time points were centrifuged at 18,000 *g*, rt for 10 min prior to LC/MS.

When determining the ideal $(MeCN)_4CuBF_4$ equiv to use for efficient DBCO protection during peptide cleavage, SPAAC was used to calculate the amount of reactive DBCO present for each sample (see **Figures 3(C)** and **S15-17**). These crude peptides were first dissolved in 5 mL 18% MeCN 0.1% TFA for LC/MS and stored at 4°C overnight. Samples were allowed to warm to rt the next day, and then SPAAC was initiated by combining 95 µL DBCO peptide solution, 95 µL 18% MeCN 0.1% TFA buffer, and 10 µL 62 mM 6-azidohexanoic acid. SPAAC reactions were placed on a rotisserie at 37°C for 1 h and then centrifuged at 18,000 *g*, rt for 10 min prior to LC/MS.

Copper Quantitation: MQuant™ Copper Test Strips

MQuant™ copper test strips were used to test aqueous peptide samples for the presence of copper (e.g., crude peptides dissolved in HPLC buffer, fractions obtained during purification). ~20 µL of aqueous sample was placed onto a test strip, and the

resulting color change was compared to the color chart on the test strip holder to obtain an approximate copper concentration. Note that the pH of HPLC buffer (0.1% TFA) is slightly lower than the lowest recommended pH in the user guide, but adjusting the pH to be within the recommended pH range did not noticeably change test strip color.

Copper Quantitation: Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Crude peptide samples were previously dissolved in 9-18% MeCN 0.1% TFA and stored in 4°C for several months. These samples were allowed to warm to rt before being centrifuged at 18,000 *g*, rt for 10 min.

HPLC-purified peptides were stored as lyophilized powder in a 4°C desiccator for several months. After allowing the powder to warm to rt, each lyophilized peptide was dissolved in 18% MeCN 0.1% TFA. The peptide solutions were then centrifuged at 18,000 *g*, rt for 10 min.

5 mL Eppendorf tubes were washed with 2.5% nitric acid in LC/MS-grade water. 2.9 mL 2.5% nitric acid in LC/MS-grade water was then added to each tube. 100 µL of each peptide sample was added to separate nitric acid solutions, and the resulting solutions were shaken by hand and vortexed to mix. Each sample was then passed through a 0.45 µm filter before heating at 95°C for 1 h (tubes left open during heating). Following the 1 h incubation, the peptide samples were taken to Dr. Diego Fernandez (ICP-MS Metals and Strontium Isotope Facility, University of Utah) for ICP-MS analyses. The amount of copper within each sample was reported in ppm.

Trypsin Digestion of Peptides and High-resolution LC/MS

~100 µg of crude, lyophilized ABCD (peptide **3b** in **Table S1**), treated with or without ~3 equiv $(MeCN)_4CuBF_4$ during cleavage, was dissolved in 720 µL trypsin

digestion buffer (40 mM ammonium bicarbonate, pH 7.8) and centrifuged for 10 min at 18,000 *g*. 360 µL supernatant was removed into a fresh Eppendorf tube before addition of 10 µL trypsin (1 mg/mL, 1:5 ratio of trypsin to peptide). Samples were then incubated for 2 h at 37°C. Trypsin was removed using Vivacon 10k MWCO filters with centrifugation at 14,000 *g* for 10 min. Flow through was collected and diluted 1:100 using LC/MS-grade 0.1% formic acid.

For MS analysis, 2 µL was injected onto a Thermo Fisher EASY-nanoLC 1000 with a Picofrit column (New Objectives, 360 µm OD x 75 µm ID, 150 mm, packed with 3 µm Reprosil-PUR) coupled to an Orbitrap Velos Pro. Mobile phases consisted of 0.1% formic acid 5% dimethylsulfoxide (DMSO) in water (Buffer A) and 0.1% formic acid 5% DMSO in MeCN (Buffer B). Gradient conditions were 5 to 45% Buffer B over 30 min at 400 nL/min. MS^1 spectra were collected using the Orbitrap analyzer from 350 to 1,550 *m/z* at 60,000 resolution (FWHM as defined at *m/z* 400). The top two most intense ions from the MS^1 scan were selected for HCD fragmentation using a normalized collision energy of 40 eV. MS^2 spectra were collected at a resolution of 15,000.

To identify peptides in an unbiased manner, raw data files were converted to .mgf format for analysis in SearchGUI[8] using the OMSSA search algorithm. Peptide sequences were added to a modified FASTA file containing ~2,000 decoy human proteins. Spectrum match settings used trypsin digestion with one missed cleavage allowed, S-tertbutylation of Cys as a fixed modification, oxidation of H, W, and M as variable modifications, precursor *m/z* tolerance of 10 ppm, and fragment *m/z* tolerance of 0.2 Da. Post-processing utilized PeptideShaker[9] to identify peptide fragments present in each sample. The false discovery rate (FDR) for peptide identification was set to

0.01. Identified peptides were exported from PeptideShaker as an MZID file to Skyline.[10] Visualization of all peptides and MS^2 spectra were produced using Skyline.

In-solution Native Chemical Ligation (NCL)

Purified N- and C-terminal peptides were dissolved in "activation buffer" (6 M GnHCl, 100 mM $NaPO_4$, pH 3) to create concentrated stocks, which could be stored at -80°C. The two peptides to be ligated by NCL were mixed in a single tube, and then diluted with activation buffer to achieve 1.28 mM N-terminal and 1.6 mM C-terminal peptide. After mixing, the solution was incubated at -20°C for 10 min. To activate the N-terminal peptide (convert the C-terminal hydrazide into an acyl azide), a small volume of 200 mM $NaNO_2$ in ddH_2O (pH adjusted to 3-4) was added to the peptide solution at rt in order to have 10 equiv $NaNO_2$ relative to the N-terminal peptide (1.2 mM N-terminal peptide, 1.5 mM C-terminal peptide, 12 mM $NaNO_2$). Upon adding $NaNO_2$, the peptide solution was quickly vortexed and put back into -20°C for 20 min. The peptide solution was then diluted 1:1 with pH 7-adjusted 300 mM MTG in "ligation buffer" (6 M GnHCl, 200 mM $NaPO_4$, pH 7) at rt. This dilution enables *in situ* thioester conversion to occur (0.6 mM N-terminal peptide, 0.75 mM C-terminal peptide, 150 mM MTG). Upon MTG addition, the pH of the reaction was quickly, but carefully to avoid thioester hydrolysis, adjusted to pH 7. The reaction was then placed on a rotisserie at rt for 10 min to enable complete thioester conversion. Due to the C-terminal peptide having an N-terminal S*t*Bu-protected Cys, pH 7-adjusted 300 mM TCEP in ligation buffer was added to the reaction after the 10 min thioester conversion. The ligation reaction (0.4 mM N-terminal peptide, 0.5 mM C-terminal peptide, 100 mM MTG, 100 mM TCEP) was rotated on a rotisserie at rt for 48 h (from MTG addition).

Time points for the in-solution NCL reactions were taken by diluting an aliquot of reaction 1:1 with 200 mM TCEP in LC/MS-grade water (pH adjusted to 7.0 prior to addition). After vortexing, acetic acid was added to a final concentration of ~5%. After vortexing the dilution again, the dilution was centrifuged at 18,000 *g*, rt for 10 min prior to HPLC and/or LC/MS analyses.

SPAAC of DBCO Peptides with Azide Peptides

Purified DBCO and azide peptides were separately dissolved in activation buffer to create concentrated stocks and stored at -80°C. The DBCO and azide peptide solutions were mixed, and activation buffer was added to dilute the solution to contain an approximately equimolar concentration of both peptides (≥1.0 mM peptide). The SPAAC reaction was placed on a rotisserie at 37°C until the reaction was complete. Time points were generally taken by diluting a reaction aliquot 1:20 with LC/MS-grade water, followed by vortexing and centrifuging the dilution at 18,000 *g*, rt for 10 min prior to HPLC and/or LC/MS. Completed reactions were frozen at -80°C prior to click-assisted NCL.

Note that if there is a significant excess of one unclicked peptide (e.g., **Figure S53**), then more of the other peptide can be added to the reaction.

Click-Assisted NCL (CAN)

Triazole-linked peptides were warmed to rt and briefly vortexed prior to being used for the CAN reaction. To perform CAN, the triazole-linked peptide was treated the same way as the combined N- and C-terminal peptide solutions used for in-solution NCL reactions. The C-terminal hydrazide was converted into an acyl azide using 10 equiv $NaNO_2$, *in situ* MTG thioester formation occurred at pH 7 with 150 mM MTG, S*t*Bu

was reduced via 100 mM TCEP addition, and time points were taken to monitor the reaction. However, the CAN reactions were much faster than the in-solution NCL's, and slightly different concentrations were used in CAN (0.5 mM, 0.05 mM, and 0.3 mM final concentration of triazole-linked peptide). In addition, CAN with 0.05 mM peptide was performed with different concentrations of MTG and TCEP (100 mM final concentration of both reagents, 10 mM MTG with 100 mM TCEP, or 10 mM both components). Once CAN reactions were complete, they were stored at -20°C or -80°C.

When performing CAN at 0.05 mM peptide concentrations, 10 mM MTG and 10 mM TCEP yielded the best results. When 100 mM MTG and 100 mM TCEP were used, we observed that a significant amount of the N-terminal Cys peptide was being capped (initially +74 Da, **Figures S39-42**). The mass of this side product suggested that modification was the result of acylation from an MTG acyl donor. This acylation occurs negligibly in the 0.5 mM CAN reactions, indicating that acylation occurs on a small amount of peptide that only becomes problematic at lower peptide concentrations. Therefore, we surmised that reducing the MTG concentration would reduce this side reaction. A 10-fold reduction in MTG concentration without reducing TCEP concentration, however, led to significant Cys desulfurization. Fortunately, a proportionate reduction of TCEP eliminated this desulfurization.

Desulfurization

"Desulfurization buffer" (5 M GnHCl, 100 mM $NaPO_4$, pH 6.5) was first sparged with argon gas for ≥ 20 min. 240 mM reduced glutathione, 120 mM VA-044 in desulfurization buffer (500 µL) was prepared, along with a solution of 550 mM TCEP in desulfurization buffer (500 µL). The finished CAN reaction was diluted 1:1 with the

reduced glutathione, VA-044 solution (440 µL was added, as there was ~440 µL of the finished CAN reaction remaining). This solution was briefly vortexed, and then 440 µL of the 550 mM TCEP buffer was added. The resulting desulfurization reaction was briefly vortexed and carefully pH adjusted to 6.5. The desulfurization reaction (0.1 mM peptide, 80 mM reduced glutathione, 40 mM VA-044, 183 mM TCEP) was then covered with argon gas and placed on a 37°C rotisserie for 16 h. Time points were taken by first diluting 60 µL desulfurization reaction in 60 µL 200 mM TCEP in LC/MS-grade water (pH adjusted to 7), vortexing, and then 6.6 µL acetic acid was added. After vortexing, the dilution was centrifuged at 18,000 *g*, rt for 10 min prior to HPLC and LC/MS analyses. The finished desulfurization reaction was stored in -80°C until Ddap cleavage.

Ddap Cleavage

Ddap cleavage buffer (~2 M hydroxylamine in desulfurization buffer, pH 6.8-6.9) was prepared by dissolving hydroxylamine in the full volume of desulfurization buffer needed (with vortexing and sonication). The buffer was then filtered (0.22 µm), and pH adjusted to 6.8-6.9 (this pH adjustment results in the hydroxylamine buffer being slightly less than 2 M). The buffer was then filtered again (0.22 µm) to avoid precipitate forming.

The Ddap cleavage reaction was initiated by diluting the peptide solution 1:1 with Ddap cleavage buffer. The reaction was then vortexed, and the pH was verified to be ~6.75. The cleavage reaction was placed on a rt rotisserie for 1.5-2 h. Time points of the Ddap cleavage reaction were taken by diluting a reaction aliquot 1:1 with 20% acetic acid in LC/MS-grade water. After vortexing the dilution, centrifugation was performed at 18,000 *g*, rt for 10 min prior to HPLC and LC/MS.

Once Ddap cleavage was complete, the entire reaction was diluted 1:1 with 20% acetic acid in LC/MS-grade water and vortexed, filtered (0.22 μm), and HPLC purified. Note that there were slight differences between the Ddap cleavages performed on peptides **9** and **17** (see **Table S1**). Peptide **9** was vortexed after thawing and immediately reacted with Ddap cleavage buffer for 2 h. Peptide **17** was centrifuged after thawing at 20,000 *g*, rt for 25 min to remove precipitate from desulfurization. The resulting supernatant was placed into a fresh tube prior to 1.5 h Ddap cleavage.

Calculation of Reaction Kinetics with GraphPad Prism

For in-solution NCL reactions, the peak areas of the reactive N-terminal peptide and ligated product were integrated for each time point using Agilent Chemstation. To normalize these peak areas, approximate extinction coefficients at 214 nm were calculated for the N-terminal peptide and ligated product, based on an established protocol[11] (reactive **5a** = 18,174 $M^{-1}cm^{-1}$; full-length **7** = 60,525 $M^{-1}cm^{-1}$; reactive **12a** = 65,954 $M^{-1}cm^{-1}$; full-length **14a**: 88,511 $M^{-1}cm^{-1}$). The concentration of ligated product was then divided by the sum of the ligated product and N-terminal peptide concentrations to provide the relative peak area of ligated material for each time point. Two independent reactions were analyzed for each in-solution NCL.

For CAN reactions, the peak areas of unligated and ligated material were integrated for each time point using Agilent Chemstation (if baselines were improperly integrated by the program, manual baseline selection was used). The extinction coefficients of unligated and ligated material should be very similar, so no adjustment was performed when comparing CAN peak areas. If S*t*Bu-protected unligated peptide peaks were observed, then the S*t*Bu-protected peptide peak area was added to the

unligated peak area (see figure legends for more details on how peaks were selected for integration). Since there was a side product with a similar retention time as unligated peptide for the 0.5 mM and 0.05 mM CAN reactions with peptide **8**, the peak area remaining at the final time point for these reactions was subtracted from the unligated area in the other time points. For each time point, the ligated product area was divided by the sum of the ligated and unligated product areas. Two independent reactions were analyzed for each CAN.

GraphPad Prism (Version 8.4.3 for Mac) was used for plotting and kinetic rate calculations shown in **Figures 6** and **10**. Note that the time points were based on when TCEP was added to the reactions in order to avoid background ligation rates from premature S*t*Bu reduction due to MTG addition. For all CAN reactions, least squares regression was used to fit a one-phase association curve to the data. No weighting was used for curve fitting, and each replicate was considered as an individual point. The curve was chosen to start at X = 0. Prism calculated the standard errors for each of the curve fit's parameters using symmetrical approximate confidence intervals. For each plot (including in-solution NCL), error bars represent standard deviation.

GraphPad Prism (Version 8.4.3 for Mac) was also used for initial rate calculations of all STEVE/KENT reactions shown in **Table S4**. Only the first two time points were analyzed for each reaction, and the times were again based on when TCEP was added to the reactions (1 and 20 min). Least squares regression was used to fit a line to the data. No weighting was used for curve fitting, and each replicate was considered as an individual point. The line was chosen to start at X = 0. Prism calculated standard errors for each of the fit line's parameters using symmetrical approximate confidence intervals.

SECTION 3: Supplemental Tables

Table S1: Summary of all peptides synthesized in this work.

Peptide	Sequence	Calculated Mass (Da)
1a	C(S*t*Bu)GK(DBCO)ENTWY-NH_2	1402.6
1b	C(S*t*Bu)GK(DBCO)ENTWY-OH	1403.6
1c	C(S*t*Bu)GK(DBCO)ENTWY-$NHNH_2$	1417.7
2a	Acetyl-RRRYSTEVEK(DBCO)NV-$NHNH_2$	1908.1
2b	Acetyl-RRRYSTEVEK(N_3)NV-$NHNH_2$	1731.9
3a	C(S*t*Bu)DEAFGHIK(DBCO)LMNPQRSTVWYK-NH_2	2926.5
3b	C(S*t*Bu)DEAFGHIKLMNPQRSTVWYK-NH_2	2611.1
3c	C(S*t*Bu)DEAFGHIK(DBCO)LNleNPQRSTVWYK-NH_2	2908.4
4a	C(S*t*Bu)GKENTWYH-NH_2	1224.4
4b	C(S*t*Bu)GKENTWYM-NH_2	1218.5
5a	Acetyl-RRRYSTEVEKNV-$NHNH_2$	1592.8
5b	Acetyl-RRRYSTEVEK(Ddap-K-N_3)NV-$NHNH_2$	2109.5
5c	Acetyl-RRRYSTEVEK(Ddap-K-PEG_8-N_3)NV-$NHNH_2$	2533.0
6a	C(S*t*Bu)GKENTWY-NH_2	1087.3
6b	C(S*t*Bu)GK(Ddap-K-DBCO)ENTWY-NH_2	1780.2
6c	C(S*t*Bu)GK(Ddap-K-PEG_8-DBCO)ENTWY-NH_2	2203.7
7	Acetyl-RRRYSTEVEKNVCGKENTWY-NH_2	2559.9
8	Acetyl-RRRYSTEVEK(Ddap-K-N_3)NV-$NHNH_2$ + C(S*t*Bu)GK(Ddap-K-DBCO)ENTWY-NH_2	3889.7

9	Acetyl-RRRYSTEVEK(Ddap-K-N_3)NVCGK(Ddap-K-DBCO)ENTWY-NH_2	3769.5
10	Acetyl-RRRYSTEVEK(Ddap-K-PEG_8-N_3)NV-$NHNH_2$ + C(S*t*Bu)GK(Ddap-K-PEG_8-DBCO)ENTWY-NH_2	4736.7
11	Acetyl-RRRYSTEVEK(Ddap-K-PEG_8-N_3)NVCGK(Ddap-K-PEG_8-DBCO)ENTWY-NH_2	4616.5
12a	AVQQNKPTRSKRGMRRSHDALTA<u>VT</u>SLSVDKTSGEKHLRHHIT-$NHNH_2$	4850.5
12b	AV**QQ**N**K**P**TRS**K**R**GNle**RR**SHDALTA<u>VT</u>SLSVDKTSGEK(Ddap-K-N_3)HL**R**HHIT-$NHNH_2$	5349.1
12c	AV**QQ**N**K**P**TRS**K(Ddap-K-N_3)**R**GNle**RR**SHDALTA<u>VT</u>SLSVDKTSGEKHL**R**HHIT-$NHNH_2$	5349.1
13a	C(S*t*Bu)DGYYRGRKVIAK-OH	1617.0
13b	C(S*t*Bu)DGYYRGRK(Ddap-K-DBCO)VIAK-OH	2309.9
14a	AVQQNKPTRSKRGMRRSHDALTAVTSLSVDKTSGEKHLRHHITCDGYYRGRKVIAK-OH	6347.3
14b	AVQQNKPTRSKRGNleRRSHDALTAVTSLSVDKTSGEKHLRHHITADGYYRGRKVIAK-OH	6297.1
15	AVQQNKPTRSKRGNleRRSHDALTAVTSLSVDKTSGEK(Ddap-K-N_3)HLRHHIT-$NHNH_2$ + C(S*t*Bu)DGYYRGRK(Ddap-K-DBCO)VIAK-OH	7659.0
16	AVQQNKPTRSKRGNleRRSHDALTAVTSLSVDKTSGEK(Ddap-K-N_3)HLRHHITCDGYYRGRK(Ddap-K-DBCO)VIAK-OH	7538.8
17	AVQQNKPTRSKRGNleRRSHDALTAVTSLSVDKTSGEK(Ddap-K-N_3)HLRHHITADGYYRGRK(Ddap-K-DBCO)VIAK-OH	7506.7
18	AVQQNKPTRSK(Ddap-K-N_3)RGNleRRSHDALTAVTSLSVDKTSGEKHLRHHIT-$NHNH_2$ + C(S*t*Bu)DGYYRGRK(Ddap-K-DBCO)VIAK-OH	7659.0
19	AVQQNKPTRSK(Ddap-K-N_3)RGNleRRSHDALTAVTSLSVDKTSGEKHLRHHITCDGYYRGRK(Ddap-K-DBCO)VIAK-OH	7538.8

Note that the N-termini of peptides **1a**, **1b**, **1c**, **3a**, **3b**, **3c**, **4a**, **4b**, **6a**, **6b**, **6c**, **12b**, **12c**, **13a**, and **13b** were capped with Boc on resin. In addition, peptides **12a**, **12b**, and **12c** were all synthesized with a VT pseudoproline during SPPS (underlined in the sequences). Finally, during SPPS of **12b** and **12c**, the bolded residues were double coupled.

Table S2: Symbols used to denote common peptide modifications.

Symbol	Modification
!	6-azidohexanoic acid triazole
#	Thioester hydrolysis
%	Met oxidation
^	MTG thioester
&	Reduction of S*t*Bu

Table S3: ICP-MS copper analysis of several peptides with or without $(MeCN)_4CuBF_4$ treatment during cleavage.

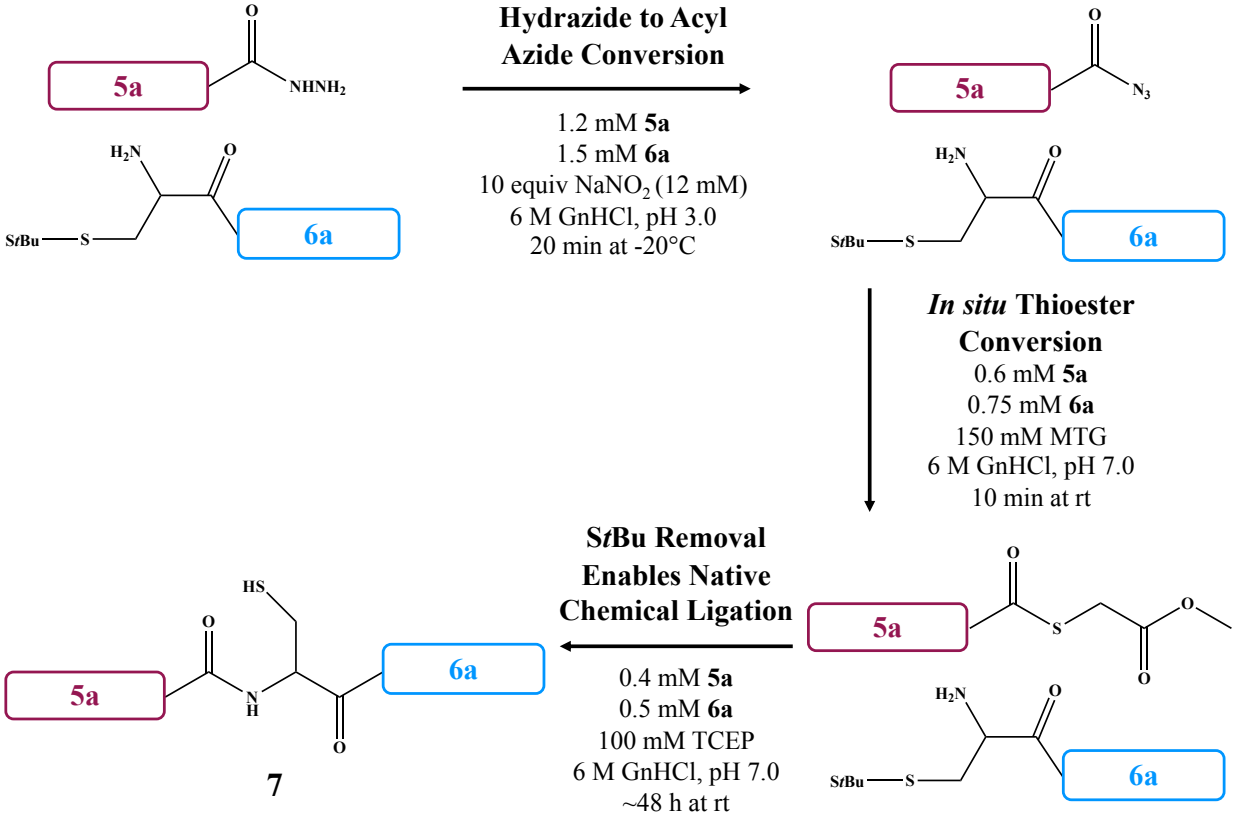
Peptide	$(MeCN)_4CuBF_4$ Equiv Used in Cleavage	HPLC Purified?	Cu (ppm)
1a	7	No	3.759
6b	2	No	0.884
5b	---	Yes	0.196
12a	---	Yes	0.204
13a	---	Yes	0.192
6b	5	Yes	0.203
6c	5	Yes	0.209
13b	5	Yes	0.196

Table S4: Initial reaction rates calculated for CAN and non-templated NCL of STEVE/KENT peptides.

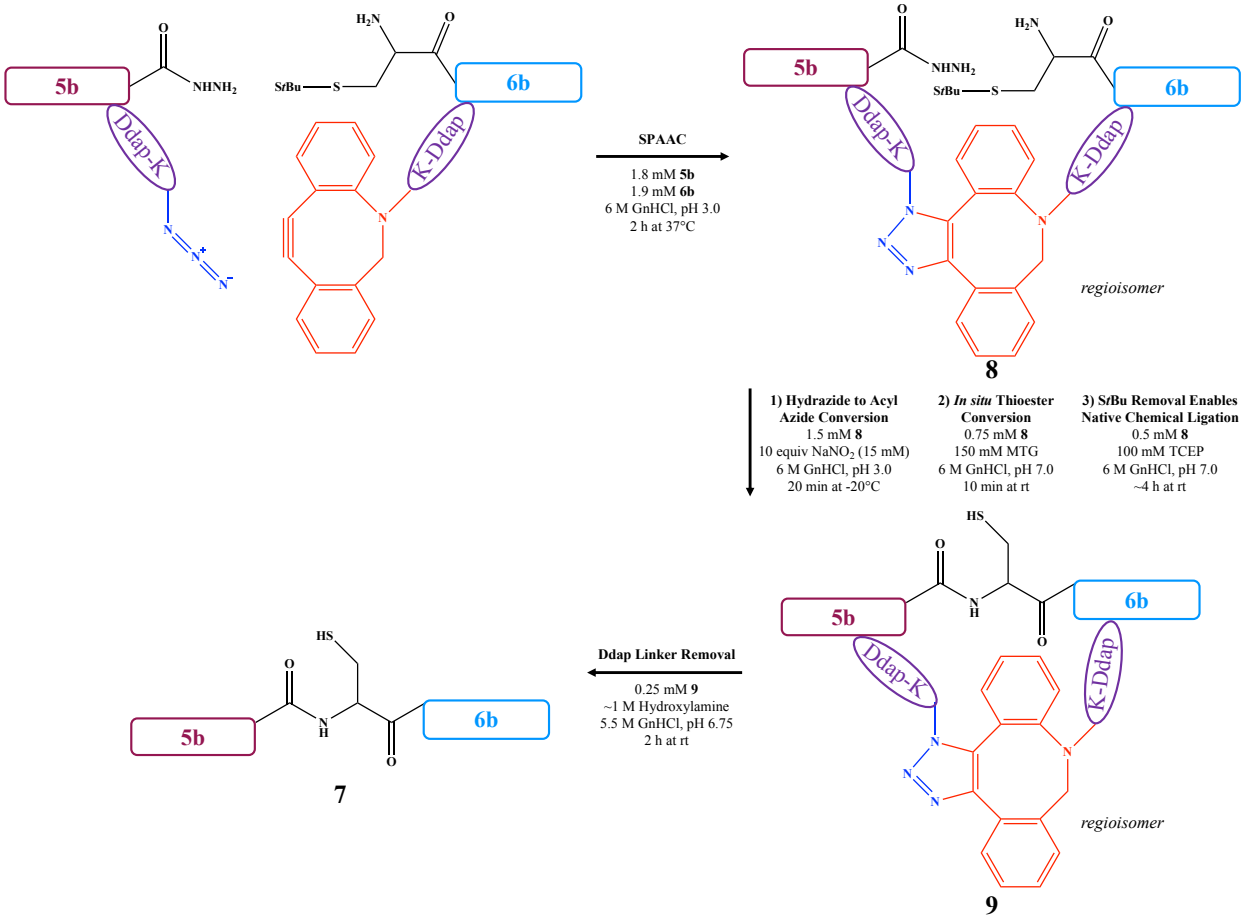
Reaction	Rate (% Ligated Product per Minute)
Without PEG_8 CAN (0.5 mM **8**)	1.6 ± 0.11
Without PEG_8 CAN (0.05 mM **8**)	1.0 ± 0.011***
With PEG_8 CAN (0.5 mM **10**)	0.59 ± 0.065
With PEG_8 CAN (0.05 mM **10**)	0.27 ± 0.038***
Non-templated NCL (0.4 mM **5a** + 0.5 mM **6a**)	0.053 ± 0.0022

Note that only the first two time points from each reaction were used to calculate initial rates. *** indicates that these initial rates are not as accurate as the other reactions, as the S*t*Bu protecting group undergoes slower deprotection in the lower concentration CAN reactions (due to the 10-fold lower TCEP concentration used). If the S*t*Bu group did not have this sluggish deprotection, then the 0.5 mM and 0.05 mM CAN reactions would be expected to have the same initial rates.

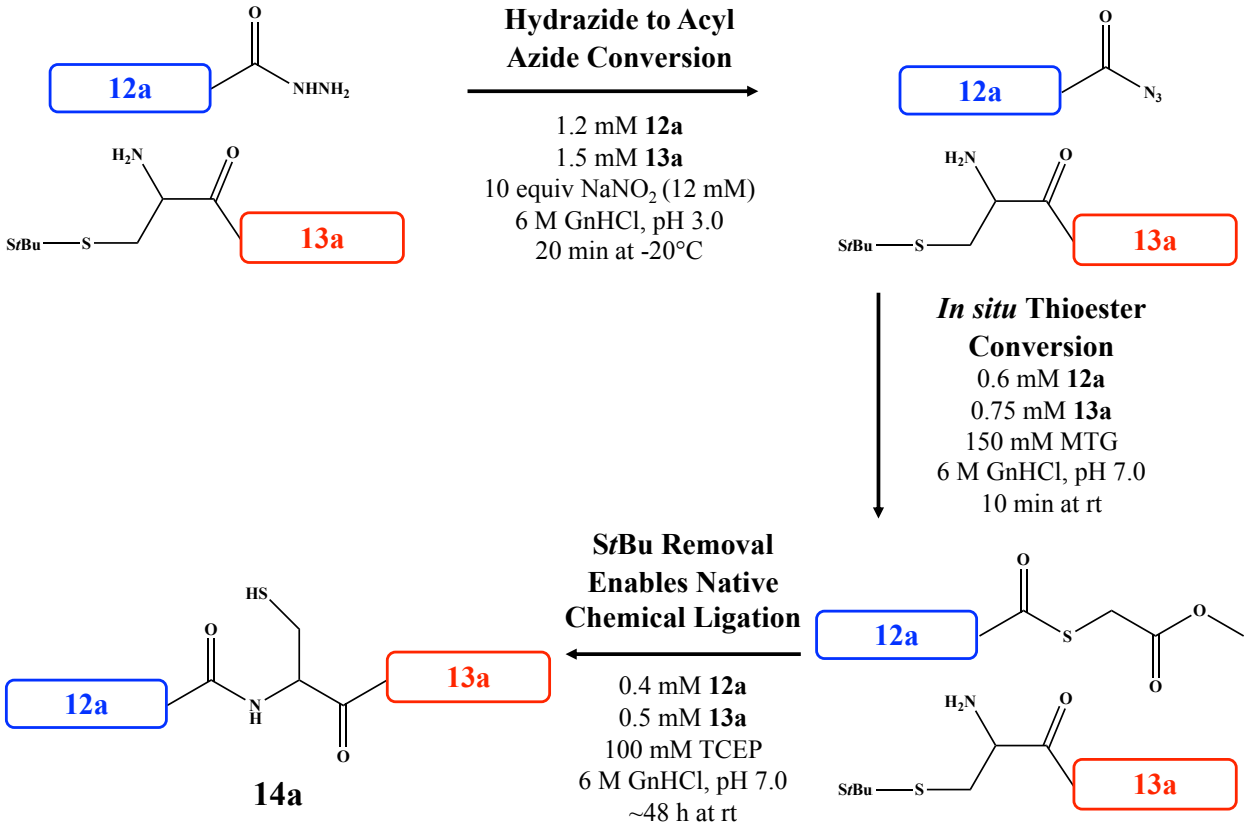
SECTION 4: Supplemental Schemes



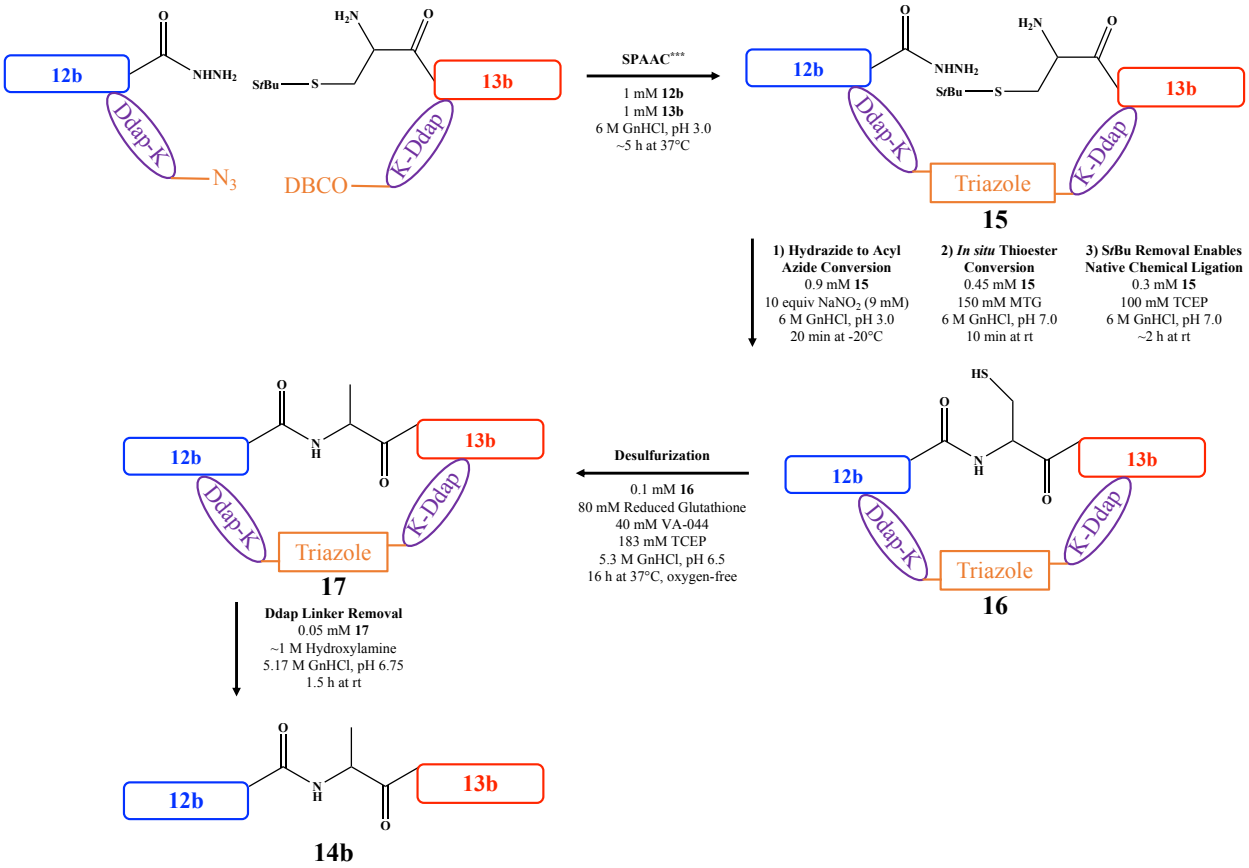
Scheme S1: Non-templated native chemical ligation between purified STEVE(Ctrl) (**5a**) and KENT(Ctrl) (**6a**) peptides. All shown steps were performed in one pot.



Scheme S2: SPAAC, click-assisted native chemical ligation, and Ddap linker removal performed with purified STEVE-Close(CAN) (**5b**) and KENT-Close(CAN) (**6b**) model peptides. All shown steps were performed in one pot.



Scheme S3: Non-templated native chemical ligation between purified L32-N(Ctrl) (**12a**) and L32-C(Ctrl) (**13a**) peptides. All shown steps were performed in one pot.



Scheme S4: SPAAC, click-assisted native chemical ligation, desulfurization, and Ddap linker removal performed on purified L32-N-Close(CAN) (**12b**) and L32-C(CAN) (**13b**) peptides. All shown steps were performed in one pot. *** Indicates that after 4 h of SPAAC at 37°C, all **12b** was reacted, but a significant amount of unreacted **13b** remained. Additional **12b** was then added to the reaction, and after 1 h at 37°C the SPAAC reaction was considered complete (though a small amount of unreacted **13b** still remained; Figures S53-54).

SECTION 5: Supplemental Figures

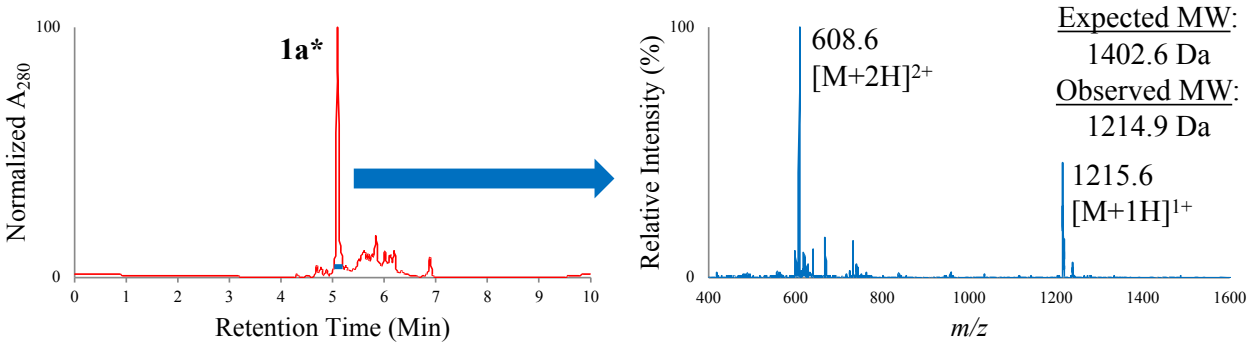


Figure S1: LC/MS analysis of crude KENT(DBCO)-NH_2 (**1a**) after standard peptide cleavage (95% TFA, 2.5% TIS, 2.5% H_2O for 3 h). LC/MS chromatogram is shown on the left, and MS of the major peak (blue bar) is shown on the right. The observed mass corresponds to 5-*endo*-*dig*-cycloisomerization of DBCO before further degradation (-187.7 Da) (**1a***). This peptide was synthesized via mini scale method A. LC/MS method A was used for analysis.

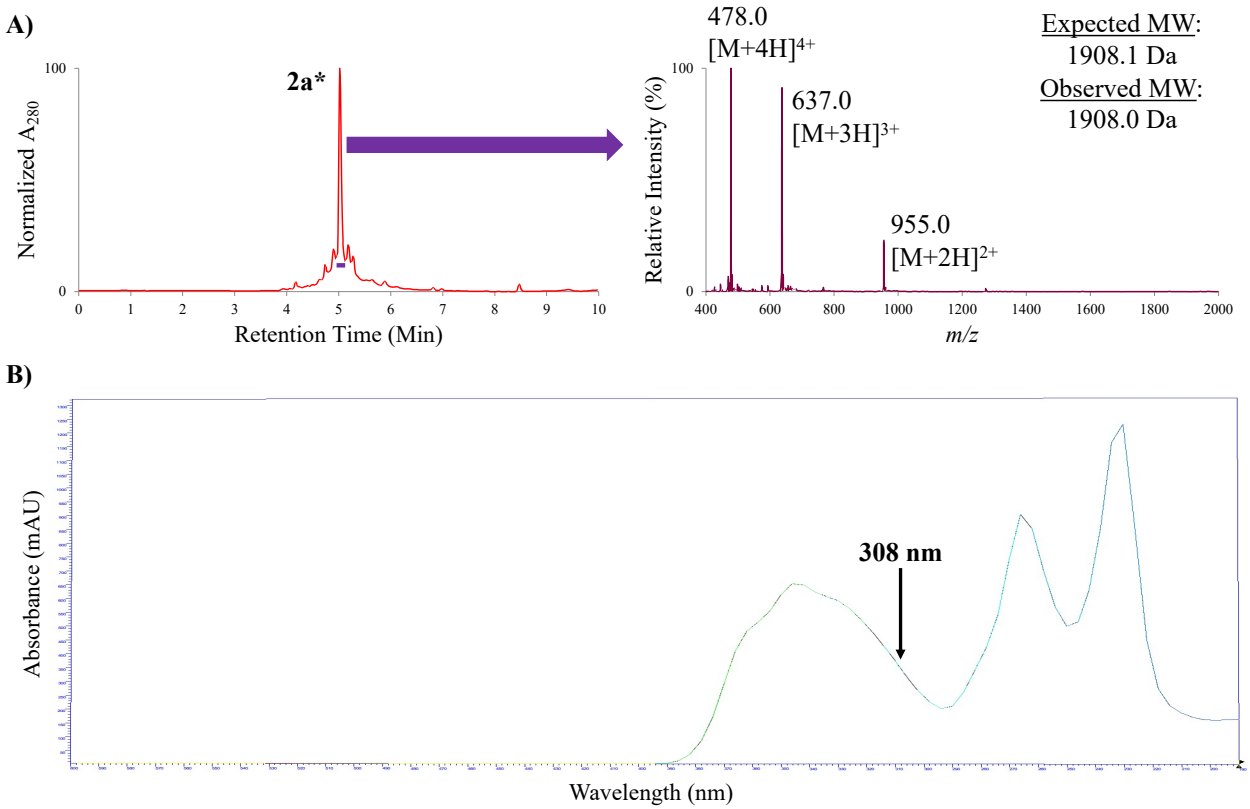


Figure S2: LC/MS analysis of crude STEVE(DBCO) (**2a**) after standard peptide cleavage. (A) LC/MS chromatogram is shown on the left, and MS of the major peak (purple bar) is shown on the right. (B) UV absorbance spectrum of the LC/MS chromatogram's major peak (5.01 – 5.03 min in part A). Although the observed mass of this peptide corresponds to the expected mass of **2a**, the UV absorbance spectrum reveals that the peptide did not have the expected local maximum absorbance near 308 nm for DBCO-containing peptides. Instead, the local maximum absorbance was shifted to a higher wavelength (~345 nm). This absorbance at higher wavelengths also interfered with the reference wavelength (360 nm) typically used as a baseline absorbance reading (the chromatogram in part A did not have a reference wavelength due to this interference). Since this peptide is unable to undergo SPAAC (Figure S3), 5-*endo*-*dig*-cycloisomerization of DBCO occurred, and the unique absorbance spectrum in part B likely indicates this ring rearrangement. As a result, * in part A indicates that DBCO on **2a** did not survive peptide cleavage. This peptide was synthesized via mini scale method A. LC/MS method A was used for analysis.

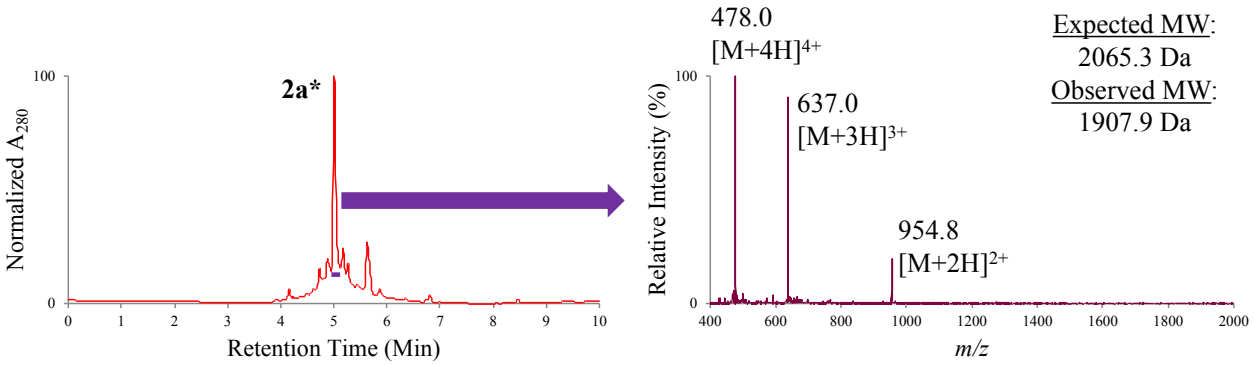


Figure S3: LC/MS analysis of the 17 h SPAAC between crude STEVE(DBCO) (**2a**) and 6-azidohexanoic acid. LC/MS chromatogram is shown on the left, and MS of the major peak (purple bar) is shown on the right. The chromatogram does not have a reference wavelength for baseline correction, based on the rearranged DBCO absorbance pattern discussed in Figure S2. * indicates that DBCO did not survive peptide cleavage, as **2a** did not undergo SPAAC (expected mass of 2065.3 Da) due to 5-*endo-dig*-cycloisomerization of DBCO. LC/MS method A was used for analysis.

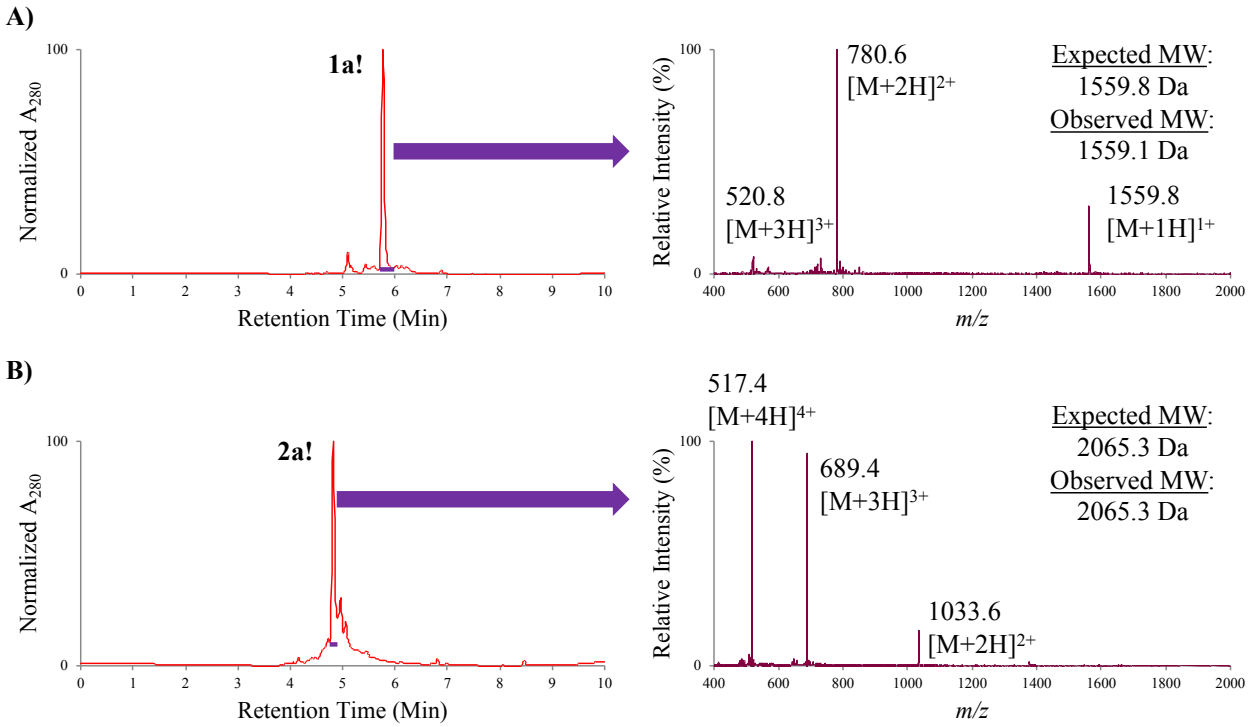


Figure S4: LC/MS analysis of DBCO-containing peptides treated with 6-azidohexanoic acid on-resin prior to cleavage under standard conditions. (A) LC/MS chromatogram of crude KENT(DBCO)-NH_2 with triazole (**1a!**) is shown on the left, and MS of the major peak (purple bar) is shown on the right. (B) LC/MS chromatogram of crude STEVE(DBCO) with triazole (**2a!**) is shown on the left, and MS of the major peak (purple bar) is shown on the right. Since on-resin SPAAC results in successful triazole formation on both peptides, the DBCO degradation observed in Figures S1-3 was from the standard peptide cleavage conditions and not the on-resin DBCO coupling procedure. Both peptides were synthesized via mini scale method A. LC/MS method A was used for both analyses.

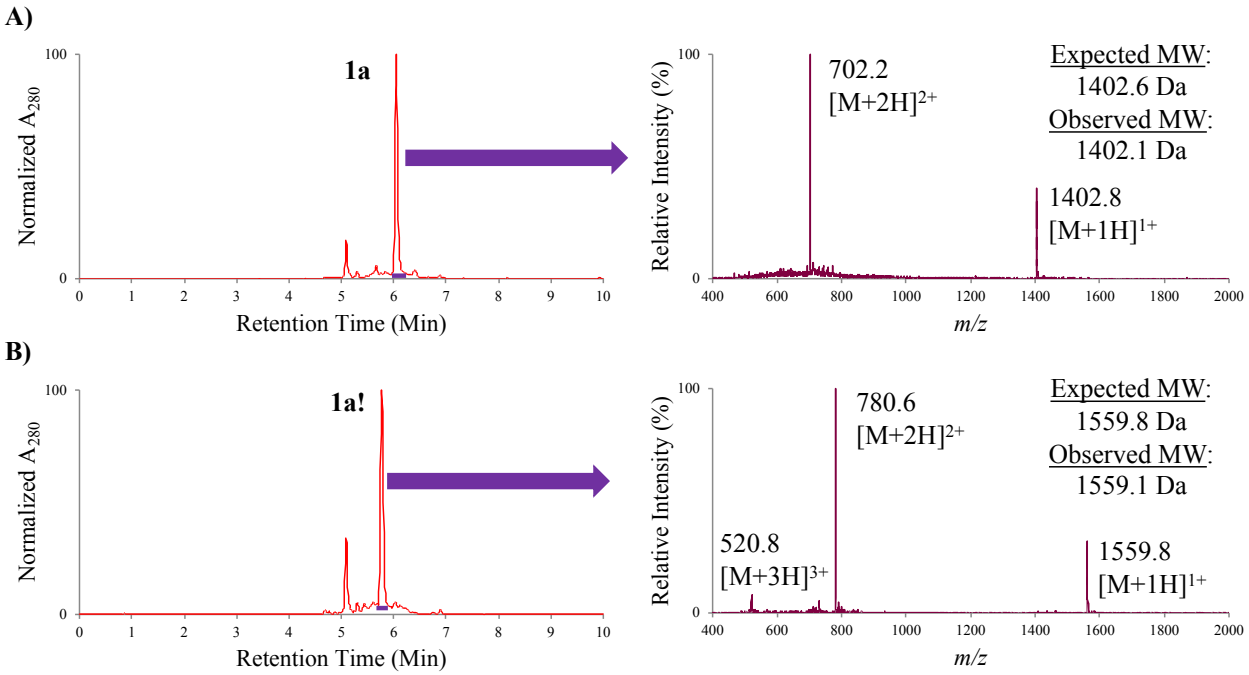


Figure S5: LC/MS analysis of KENT(DBCO)-NH_2 (**1a**) treated with $(MeCN)_4CuBF_4$ dissolved in DMF for 1 h prior to cleavage under standard conditions. (A) LC/MS chromatogram of crude **1a** is shown on the left, and MS of the major peak (purple bar) is shown on the right. (B) LC/MS chromatogram of KENT(DBCO)-NH_2 with triazole (**1a!**) after 18 h SPAAC between crude **1a** and 6-azidohexanoic acid. MS of the major peak (purple bar) is shown on the right. These results indicate that the $(MeCN)_4CuBF_4$ treatment successfully protects **1a** from DBCO degradation during peptide cleavage. This peptide was synthesized via mini scale method A. LC/MS method A was used for analyses.

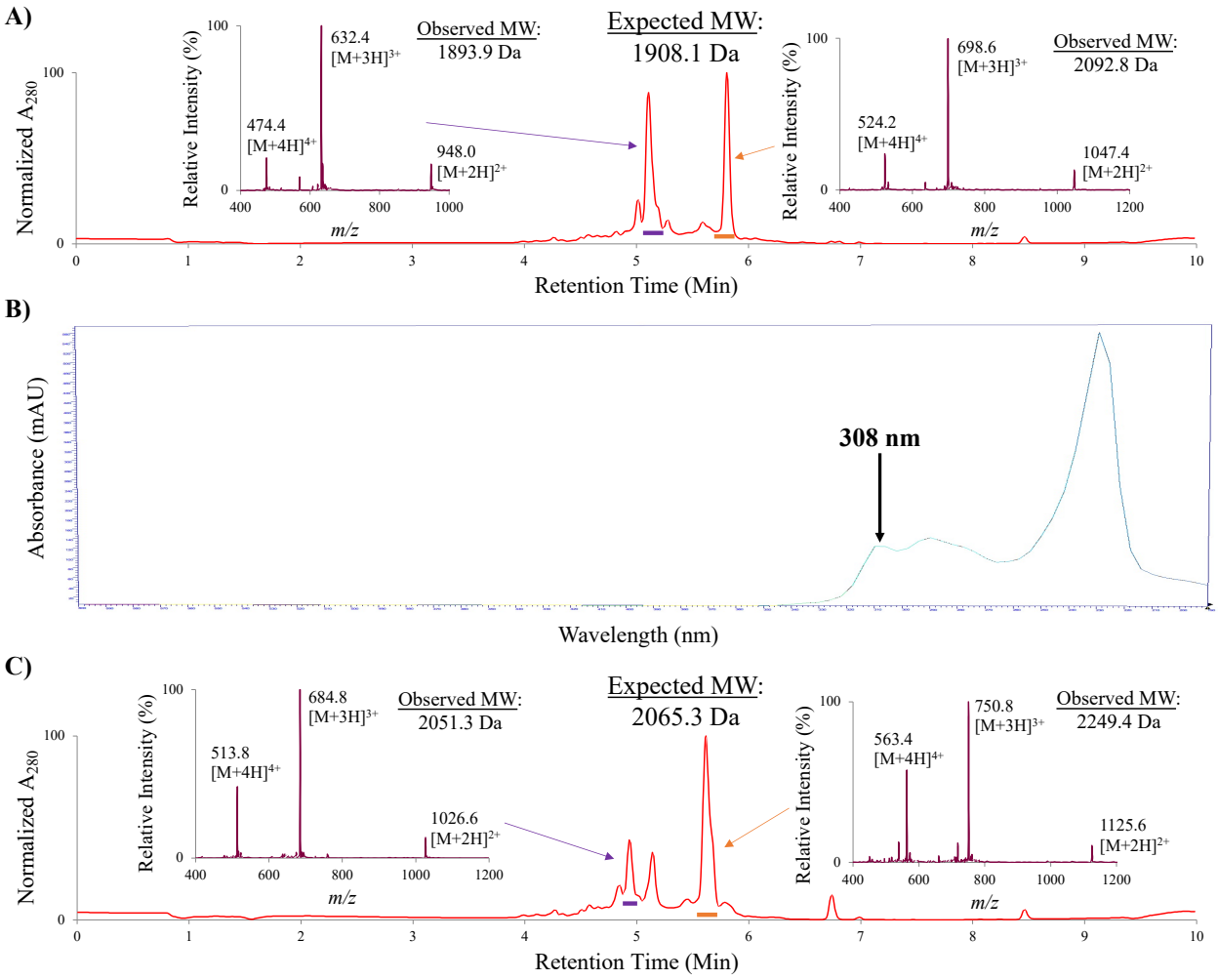


Figure S6: LC/MS analysis of STEVE(DBCO) (**2a**) treated with $(MeCN)_4CuBF_4$ dissolved in DMF for 1 h prior to cleavage under standard conditions. (A) LC/MS chromatogram of crude **2a**. MS of the major peaks (purple and orange bars) are shown. The expected mass of **2a** was not observed within either of these peaks. (B) UV absorbance spectrum of the LC/MS chromatogram's peak with the purple arrow (5.10 – 5.12 min in part A). This peak has a local maximum absorbance near 308 nm, unlike for rearranged DBCO (Figure S2). Note that the UV absorbance spectrum of the LC/MS chromatogram's peak with the orange arrow also has the local maximum absorbance near 308 nm (not shown). (C) LC/MS chromatogram of the 17 h SPAAC between crude **2a** and 6-azidohexanoic acid. MS of the major peaks (purple and orange bars) are shown. Observed masses indicate that reactive DBCO is present in crude **2a**, as both of the highlighted peaks demonstrate successful triazole formation during SPAAC (+~157.2 Da compared to the observed masses shown in part A). Overall, these results indicate that the $(MeCN)_4CuBF_4$ treatment successfully protects **2a** from DBCO degradation during peptide cleavage, but this treatment causes side reactions with the 2-chlorotrityl chloride resin and/or the C-terminal hydrazide. Note that switching the solvents used to dissolve $(MeCN)_4CuBF_4$ (DCM, N-methylpyrrolidone, methanol, and MeCN) did not overcome these side reactions (data not shown). This peptide was synthesized via mini scale method A. LC/MS method A was used for analyses.

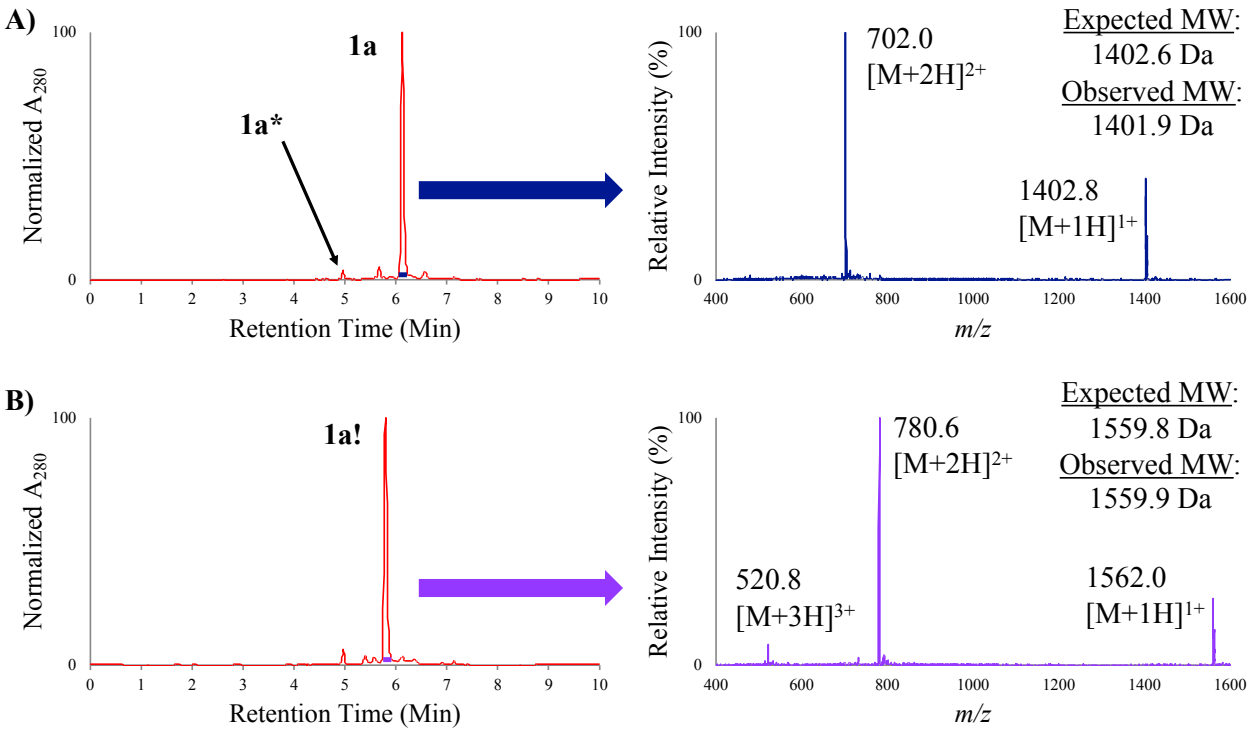


Figure S7: LC/MS analysis of KENT(DBCO)-NH_2 (**1a**) cleaved with ~1.5 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. (A) LC/MS chromatogram of crude **1a** is shown on the left, and MS of the major peak (blue bar) is shown on the right. **1a*** corresponds to **1a** that underwent 5-*endo*-*dig*-cycloisomerization of DBCO before further degradation (-188 Da). (B) LC/MS chromatogram of KENT(DBCO)-NH_2 with triazole (**1a!**) after 16 h SPAAC between crude **1a** and 6-azidohexanoic acid. MS of the major peak (magenta bar) is shown on the right. These results indicate that adding $(MeCN)_4CuBF_4$ directly to the standard cleavage cocktail protects **1a** from acid-mediated DBCO degradation. Note that Figure 2 shows the overlaid spectra for both of these chromatograms in order to highlight the retention time shift of **1a** compared to **1a!**. The deconvoluted MS of **1a!** shown in Figure 2 was generated with the UniDec software.[7] This peptide was synthesized via mini scale method A. LC/MS method C was used for analyses.

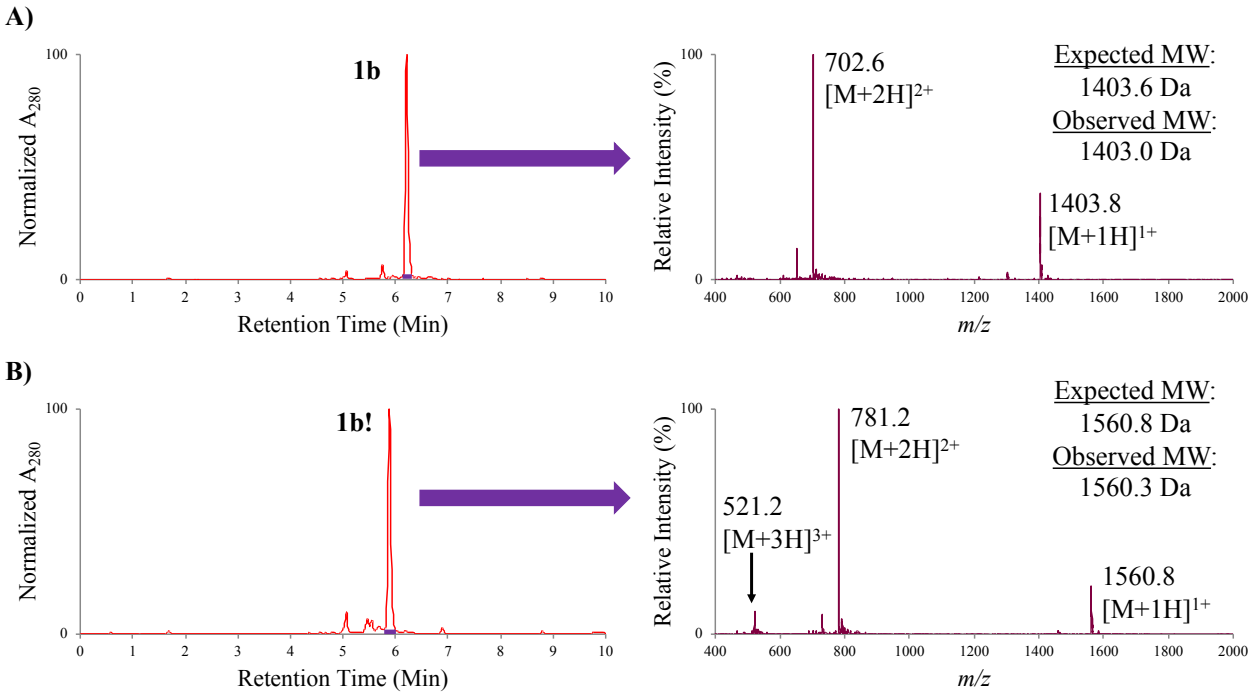


Figure S8: LC/MS analysis of KENT(DBCO)-OH (**1b**) cleaved with ~1.5 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. (A) LC/MS chromatogram of crude **1b** is shown on the left, and MS of the major peak (purple bar) is shown on the right. (B) LC/MS chromatogram of KENT(DBCO)-OH with triazole (**1b!**) after 26 h SPAAC between crude **1b** and 6-azidohexanoic acid. MS of the major peak (purple bar) is shown on the right. These results indicate that adding $(MeCN)_4CuBF_4$ directly to the standard cleavage cocktail protects **1b** from acid-mediated DBCO degradation. This peptide was synthesized via mini scale method A. LC/MS method C was used for analyses.

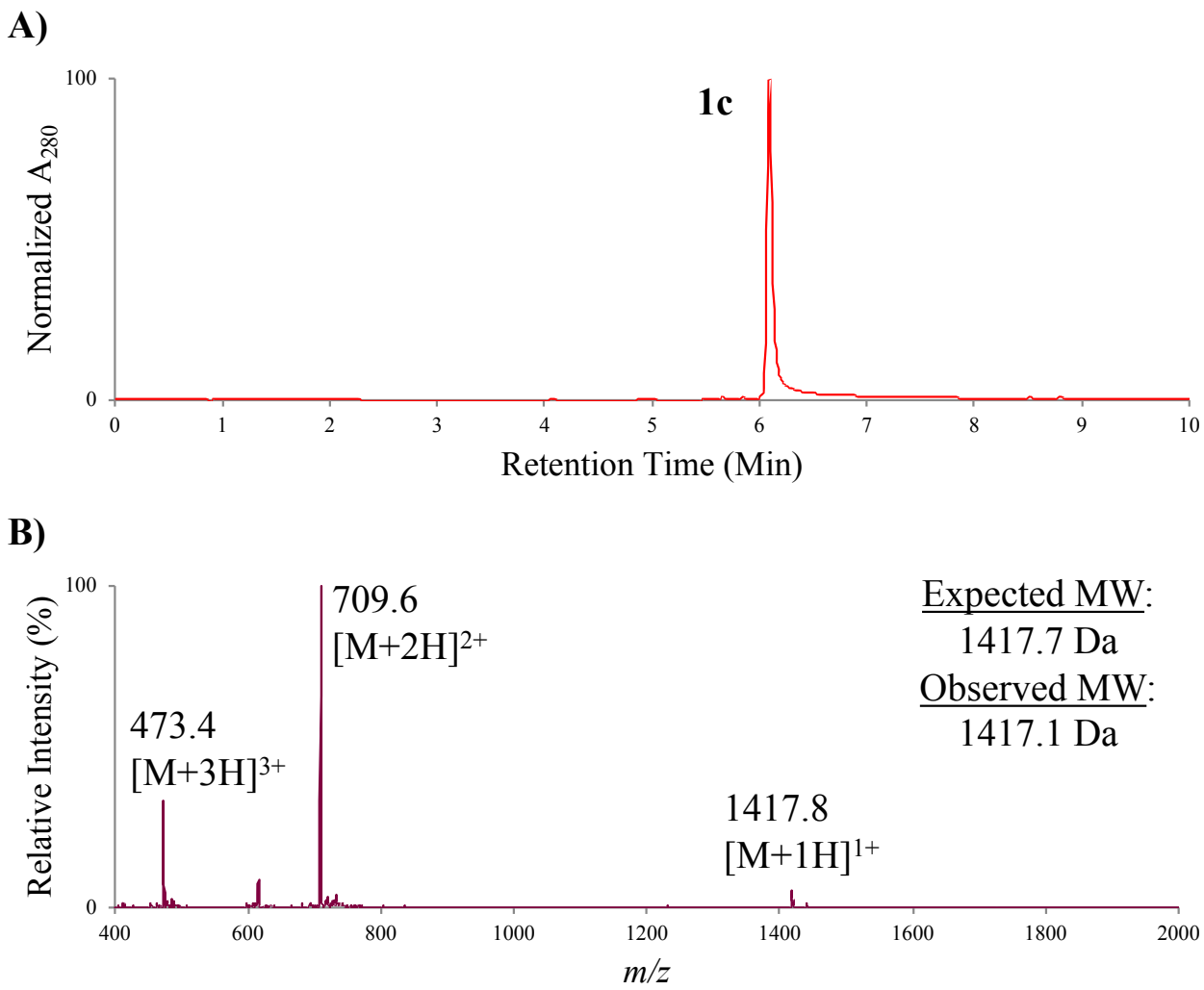


Figure S9: Pure KENT(DBCO)-$NHNH_2$ (**1c**) after HPLC purification to determine if the leftover $(MeCN)_4CuBF_4$ from cleavage would be removed. (A) LC/MS chromatogram. (B) MS for the entire chromatogram shown in part A (2 – 10 min, as the first 2 min are not injected into the mass spectrometer). Copper test strips used on pure **1c** indicated that copper was not present, suggesting that HPLC purification successfully removes copper from the peptide. Note that this peptide was synthesized via a 4.5x mini scale method A (i.e., 4.5x resin was weighed out for manual syntheses and 4.5x solution volumes were used for couplings and cleavages). ~1.5 equiv $(MeCN)_4CuBF_4$ was used with standard cleavage conditions. This peptide was purified using purification methods A-C. LC/MS method C was used for analysis.

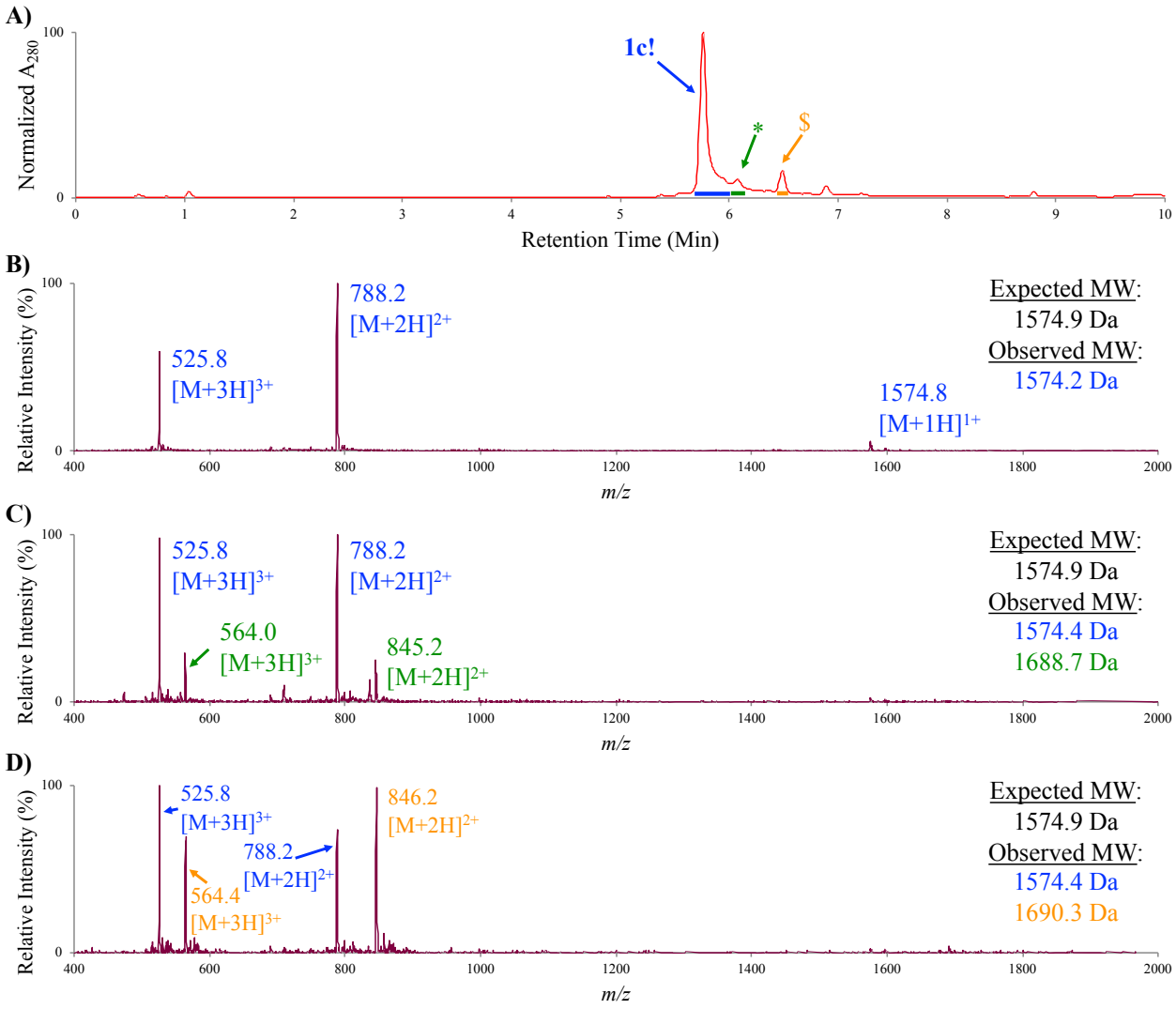


Figure S10: LC/MS analysis of the 2 h time point from the SPAAC between pure KENT(DBCO)-$NHNH_2$ (**1c**) and 6-azidohexanoic acid (124 µM **1c** and 231 µM 6-azidohexanoic acid in 18% MeCN 0.1% TFA, 37°C). (A) LC/MS chromatogram. The reaction was complete after 2 h, as unreacted **1c** was no longer observed, and the expected KENT(DBCO)-$NHNH_2$ with triazole (**1c!**) successfully formed. * and $ refer to minor side products that formed during the reaction. (B) MS of **1c!** (blue bar in part A). (C) MS of the first side product (*, green bar in part A). The mass written in green text corresponds to the observed side product, as the **1c!** mass is likely from the peptide tailing off of the LC column (commonly observed with C-terminal hydrazide peptides). (D) MS of the second side product ($, orange bar in part A). The mass written in orange text corresponds to the observed side product, as the **1c!** mass is likely from the peptide tailing off of the LC column. Overall, this data indicates that pure **1c** successfully undergoes SPAAC to form **1c!**. Although crude **1c** also undergoes SPAAC (not shown), a significant amount of side products also form, likely from residual $(MeCN)_4CuBF_4$ in the crude peptide. Therefore, HPLC purification of DBCO C-terminal hydrazide peptides is required to enable efficient SPAAC, as residual $(MeCN)_4CuBF_4$ is removed (Figure S9 and Table S3). LC/MS method C was used for analysis.

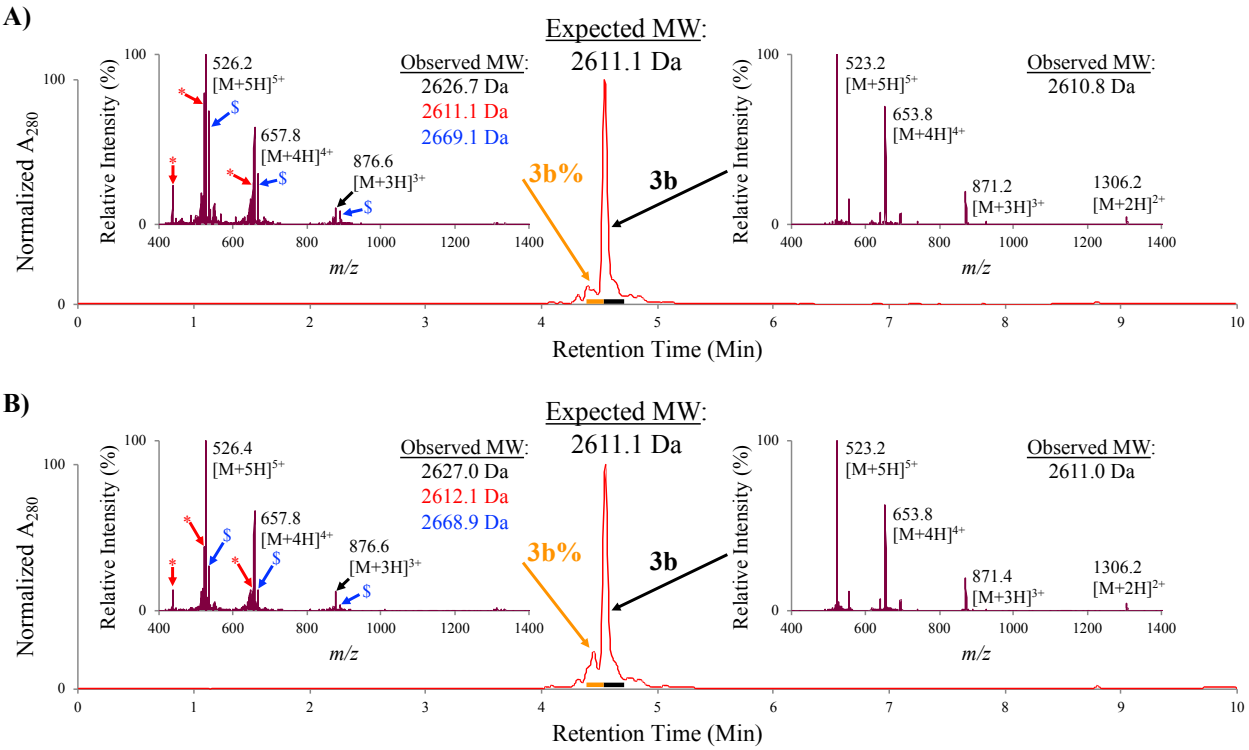


Figure S11: LC/MS analysis of ABCD (**3b**) cleaved with ± ~3 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. (A) LC/MS chromatogram of crude **3b** following standard cleavage without $(MeCN)_4CuBF_4$. MS of the major peaks (orange and black bars) are shown. Nearly all of the crude material corresponds to **3b** (observed mass of 2610.8 Da). A small amount of side product also formed, as indicated by the MS of the peak with the orange arrow. The majority of this side product is oxidized **3b** (**3b%**, mass of 2626.7 Da), and a small amount of unknown side product is also observed ($ in the MS, mass of 2669.1 Da). (B) LC/MS chromatogram of crude **3b** following standard cleavage with ~3 equiv $(MeCN)_4CuBF_4$. MS of the major peaks (orange and black bars) are shown. The crude material is similar to part A, except more **3b%** is observed when $(MeCN)_4CuBF_4$ is included during cleavage (based on peak areas and the increased relative amount of **3b%** ions in the MS). Note that Figure 3(A) shows the overlaid spectra for both of these chromatograms in order to highlight the increased formation of **3b%** when $(MeCN)_4CuBF_4$ is used during cleavage. These peptides were synthesized via mini scale method A. LC/MS method C was used for analyses.

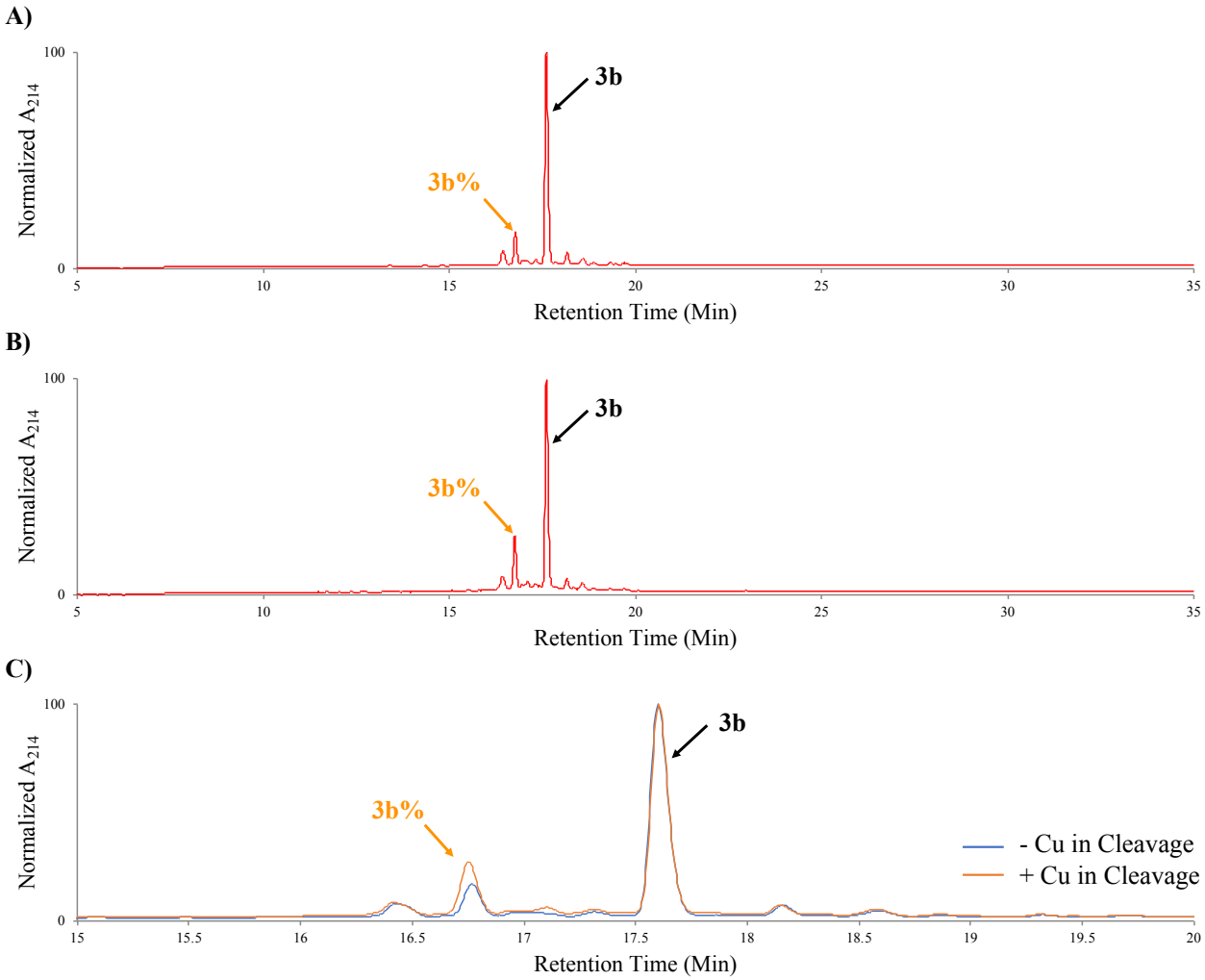


Figure S12: HPLC analysis of ABCD (**3b**) cleaved with ± ~3 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. Note that these peptides are the same crude **3b** peptides used for Figures 3(A-B) and S11. (A) HPLC chromatogram of crude **3b** following standard cleavage without $(MeCN)_4CuBF_4$. (B) HPLC chromatogram of crude **3b** following standard cleavage with ~3 equiv of $(MeCN)_4CuBF_4$. (C) Overlaid HPLC chromatograms of parts A and B. Based on the peak areas of **3b** and oxidized **3b** (**3b%**), 12.7% of crude material was oxidized in part A, whereas 19.7% was oxidized in part B. Note that these percentages are slightly higher than those calculated via LC/MS analysis (Figure 3(A)). This is likely due to oxidation occurring specifically during HPLC analysis, as we have observed several peptides that show much more oxidation when TFA is used in chromatography (HPLC) compared to when formic acid is used (LC/MS). Analytical method A was used for HPLC analyses.

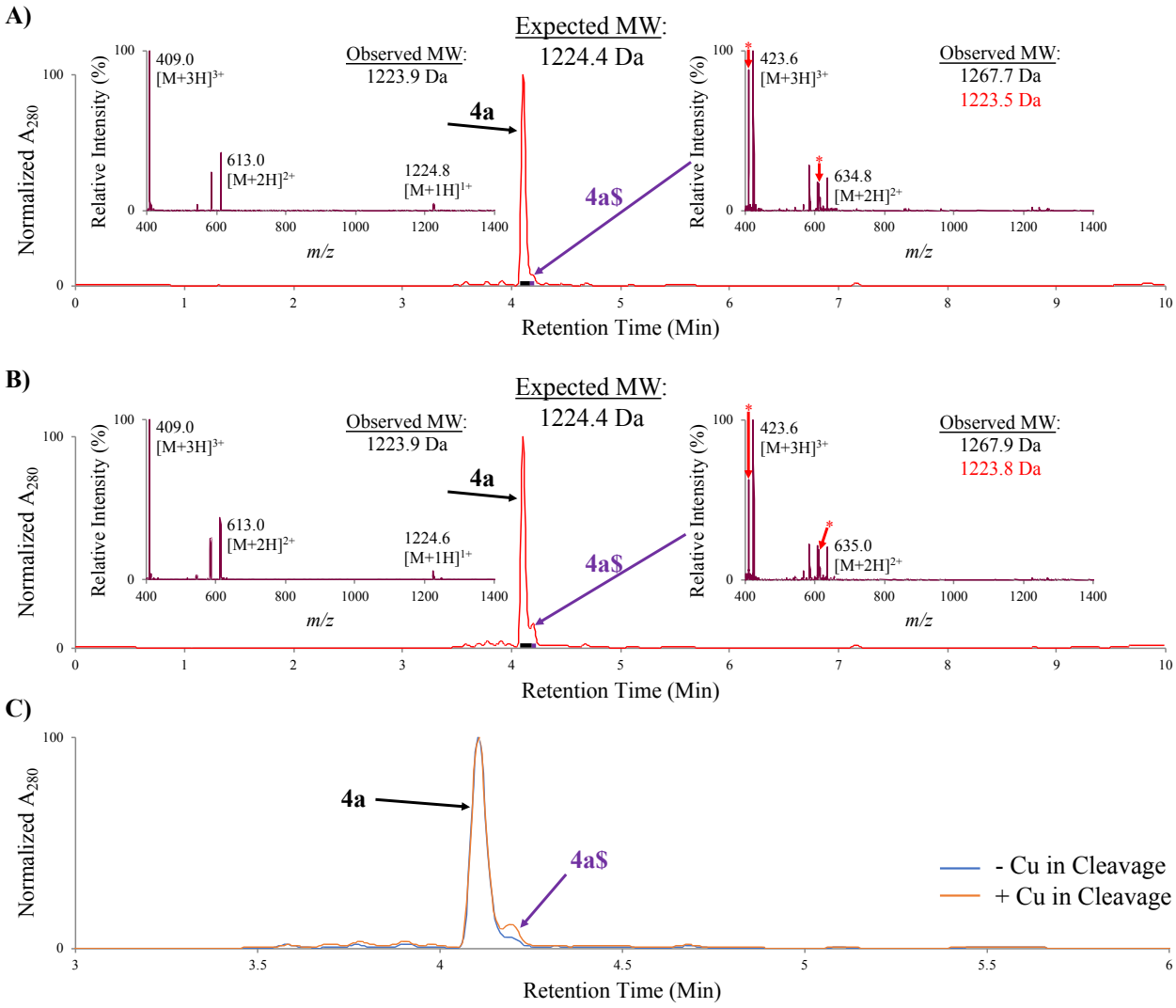


Figure S13: LC/MS analysis of KENT-H (**4a**) cleaved with ± ~1.5 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. (A) LC/MS chromatogram of crude **4a** following standard cleavage without $(MeCN)_4CuBF_4$. MS of the major peaks (black and purple bars) are shown. Most of the crude material corresponds to **4a**, and a small amount of side product also formed (incomplete Trp deprotection (**4a$**), observed mass of 1267.7 Da). (B) LC/MS chromatogram of crude **4a** following standard cleavage with ~1.5 equiv $(MeCN)_4CuBF_4$. MS of the major peaks (black and purple bars) are shown. The crude material is similar to part A, but the incomplete Trp deprotection side product (**4a$**) increased slightly. (C) Overlaid LC/MS chromatograms of parts A and B. The increased amount of **4a$** is not likely caused by $(MeCN)_4CuBF_4$ being used in the cleavage, as this incomplete Trp deprotection is commonly observed within crude peptides. Importantly, addition of $(MeCN)_4CuBF_4$ to the cleavage did not result in increased KENT-H oxidation, suggesting that His is not susceptible to oxidation during cleavage with copper. These peptides were synthesized via mini scale method A. LC/MS method C was used for analyses.

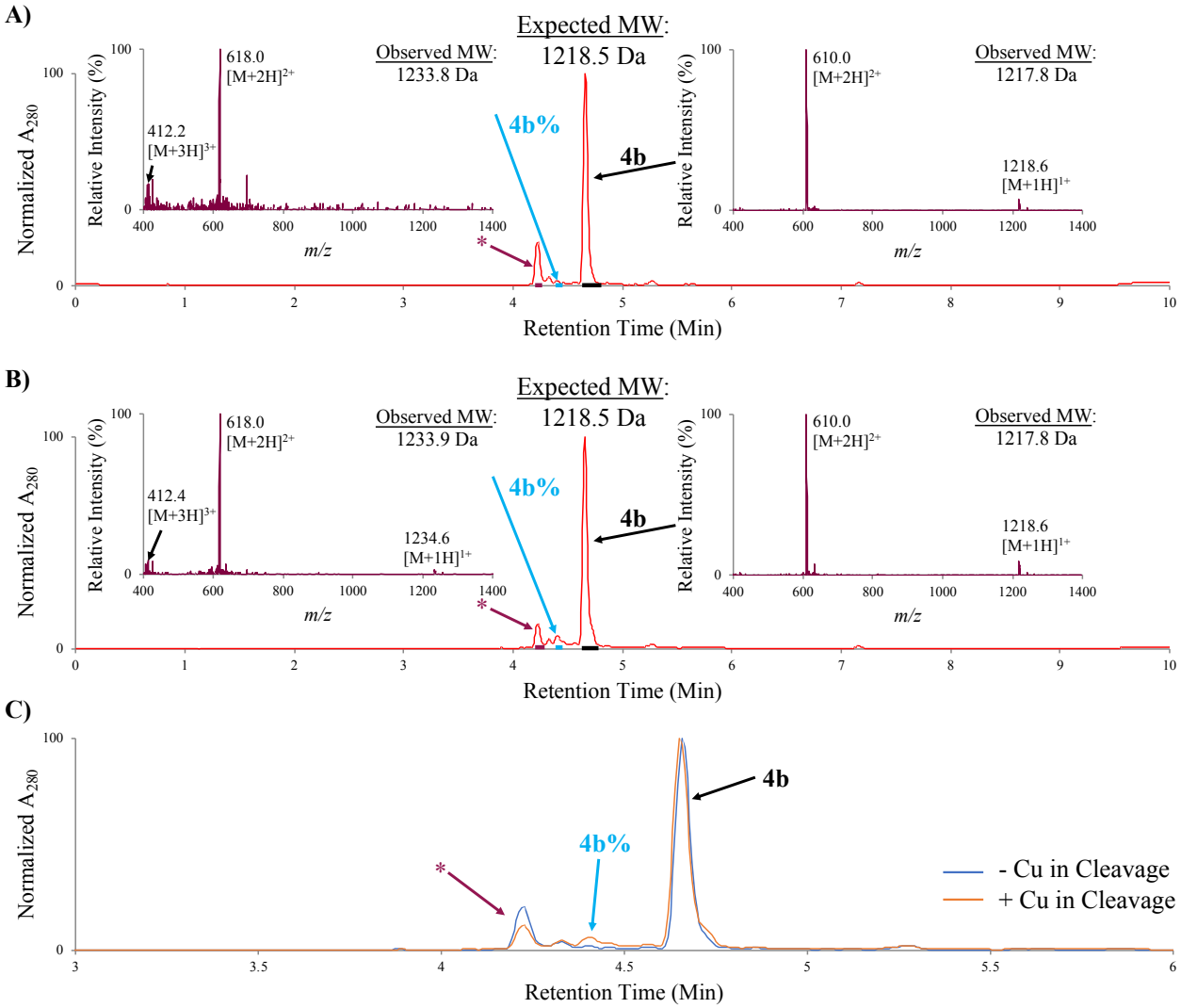


Figure S14: LC/MS analysis of KENT-M (**4b**) cleaved with ± ~1.5 equiv $(MeCN)_4CuBF_4$ in the standard cleavage cocktail. (A) LC/MS chromatogram of crude **4b** following standard cleavage without $(MeCN)_4CuBF_4$. MS of the important peaks (cyan and black bars) are shown. Most of the crude material corresponds to **4b**, and some peptide with incomplete *t*Bu deprotection formed (*, observed mass of 1273.9 Da). A negligible amount of oxidized **4b** (**4b%**) also formed. (B) LC/MS chromatogram of crude **4b** following standard cleavage with ~1.5 equiv $(MeCN)_4CuBF_4$. MS of the important peaks (cyan and black bars) are shown. The crude material is similar to part A. However, an increased amount of **4b%** was observed, and a decreased amount of incomplete *t*Bu deprotection (*) also occurred. (C) Overlaid LC/MS chromatograms of parts A and B. The decreased amount of * is not likely caused by $(MeCN)_4CuBF_4$ addition to the cleavage, as this incomplete *t*Bu deprotection is commonly observed after peptide cleavages. However, the increased amount of **4b%** is likely directly caused by $(MeCN)_4CuBF_4$ addition, as copper is known to oxidize certain amino acids, and oxidation is observed in the ABCD (**3b**) peptide (Figures S11-12). Since oxidation occurred in **4b** but not **4a** (Figure S13), $(MeCN)_4CuBF_4$-catalyzed oxidation is likely specific to Met during peptide cleavage. These peptides were synthesized via mini scale method A. LC/MS method C was used.

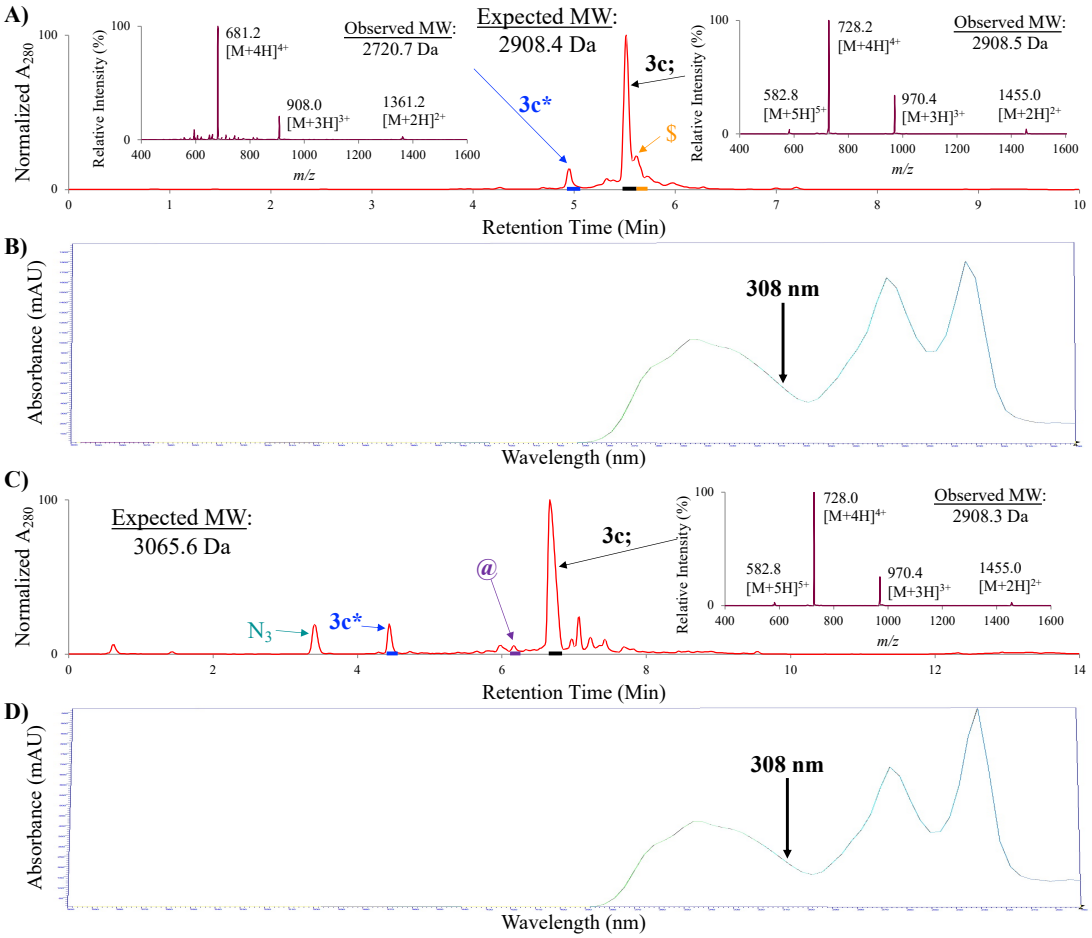


Figure S15: LC/MS analysis of ABCD-Nle(DBCO) (**3c**) cleaved under standard conditions without $(MeCN)_4CuBF_4$. (A) LC/MS chromatogram of crude **3c**. MS of the major peaks (blue and black bars) are shown. Although the mass of the largest peak corresponds to the expected mass for **3c** (2908.5 Da), this is likely peptide that underwent 5-*endo*-*dig*-cycloisomerization of DBCO (**3c;**) since $(MeCN)_4CuBF_4$ was not present during cleavage to protect DBCO. Degraded DBCO peptide (**3c***, mass is -188 Da compared to the expected mass) is also observed. $ refers to a mass of 2908.4 Da, which may be an isomer of **3c;**. (B) UV absorbance spectrum of the LC/MS chromatogram's largest peak (5.50 – 5.52 min in part A). Since the expected local maximum absorbance of DBCO is not observed near 308 nm, DBCO in **3c** likely underwent 5-*endo*-*dig*-cycloisomerization (see Figure S2). (C) LC/MS chromatogram of the 1 h SPAAC between crude **3c** and 6-azidohexanoic acid. MS of the largest peak (black bar) is shown. As expected, most of the chromatogram corresponds to **3c** that underwent 5-*endo*-*dig*-cycloisomerization of DBCO (**3c;**) during cleavage. Degraded DBCO peptide (**3c***, mass of 2720.8 Da is -188 Da compared to the expected mass of **3c**) is still observed. @ refers to the peak considered to be ABCD-Nle(DBCO) with triazole. MS of this peak did not show ions corresponding to the triazole product, but peaks at this retention time have the triazole peptide in the other SPAAC reactions (e.g., Figures S16-17). N_3 refers to 6-azidohexanoic acid. To calculate the amount of reactive DBCO shown in Figure 3(C), the peak area of @ was divided by the total peak area of @, **3c***, and **3c;**, and the resulting quotient was multiplied by 100. (D) UV absorbance spectrum of the LC/MS

chromatogram's largest peak (6.66 – 6.68 min in part C). This spectrum looks like part B, further indicating that the DBCO in **3c** underwent 5-*endo*-*dig*-cycloisomerization, inhibiting reaction with 6-azidohexanoic acid. Note that the chromatograms in parts A and C do not have a reference wavelength for baseline correction, based on rearranged DBCO's absorbance interfering with this baseline measurement (discussed in Figure S2). This peptide was synthesized via mini scale method B. LC/MS method C was used for parts A and B, while LC/MS method D was used for parts C and D.

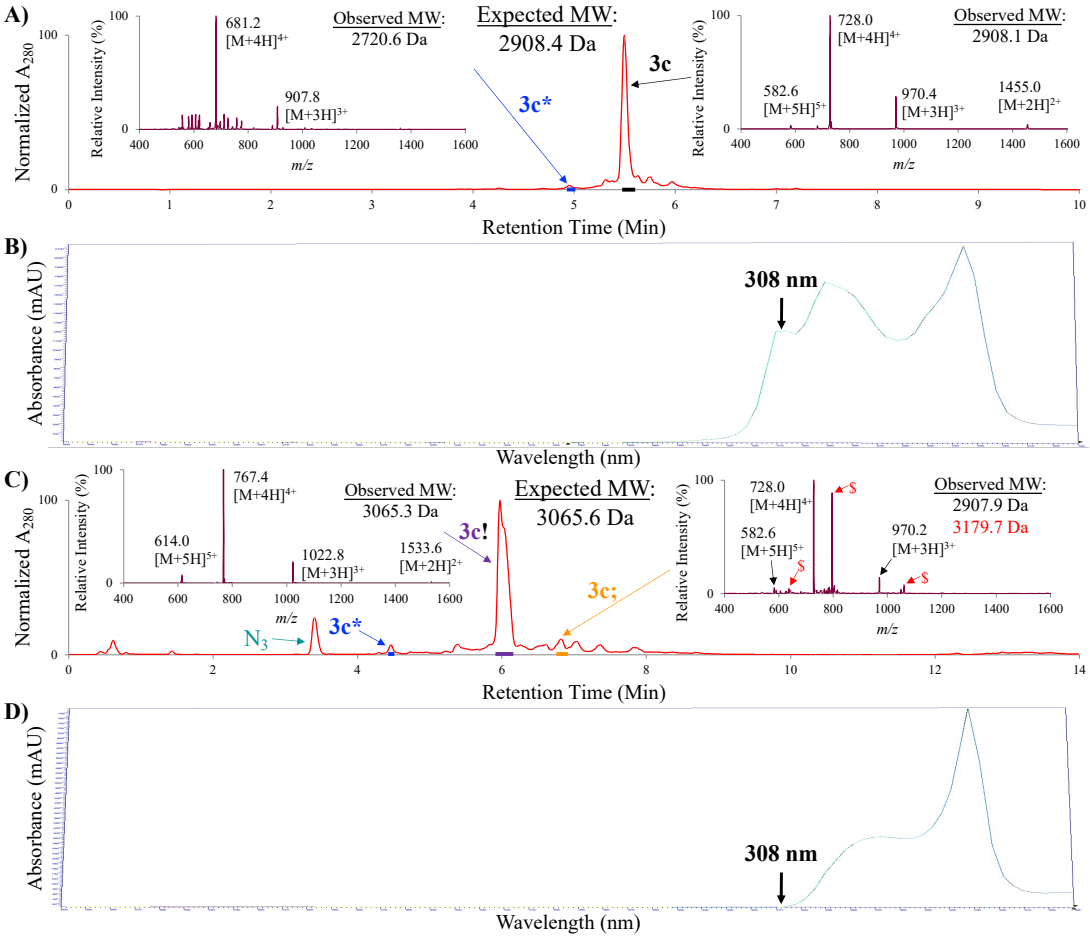


Figure S16: LC/MS analysis of ABCD-Nle(DBCO) (**3c**) cleaved under standard conditions with 5 equiv of $(MeCN)_4CuBF_4$. (A) LC/MS chromatogram of crude **3c**. MS of the major peaks (blue and black bars) are shown. Most of the crude material corresponds to **3c**, and a small amount of degraded DBCO peptide (**3c***, mass is -188 Da compared to the expected mass) is also observed. (B) UV absorbance spectrum of the LC/MS chromatogram's largest peak (5.49 – 5.51 min in part A). As expected for DBCO peptides, a local maximum absorbance is observed near 308 nm. This absorbance pattern indicates that most of the DBCO in the **3c** peak is intact. (C) LC/MS chromatogram of the 1 h SPAAC between crude **3c** and 6-azidohexanoic acid. MS of two major peaks (purple and orange bars) are shown. The largest peak corresponds to ABCD-Nle(DBCO) with triazole (**3c!**). A smaller peak corresponds to **3c** that underwent 5-*endo-dig*-cycloisomerization of DBCO (**3c;**) during cleavage, based on the observed mass matching **3c** and the UV absorbance spectrum (not shown) appearing similar to those in Figures S2 and S15. However, an unknown side product was also observed in the **3c;** MS (ions with $ and mass written in red text). Degraded DBCO peptide (**3c***, mass of 2721.2 Da is -187 Da compared to the expected mass of **3c**) is still observed. N_3 refers to 6-azidohexanoic acid. To calculate the amount of reactive DBCO shown in Figure 3(C), the peak area of **3c!** was divided by the total peak area of **3c!**, **3c***, and **3c;**, and the resulting quotient was multiplied by 100. (D) UV absorbance spectrum of the LC/MS chromatogram's largest peak (5.99 – 6.01 min in part C). There is no absorbance detected near 308 nm, as expected for DBCO peptides that successfully formed triazole with 6-

azidohexanoic acid. Note that the chromatograms in parts A and C do not have a reference wavelength for baseline correction, based on the potential for rearranged DBCO's absorbance to interfere with this baseline measurement (discussed in Figure S2). This peptide was synthesized via mini scale method B. LC/MS method C was used for parts A and B, while LC/MS method D was used for parts C and D.

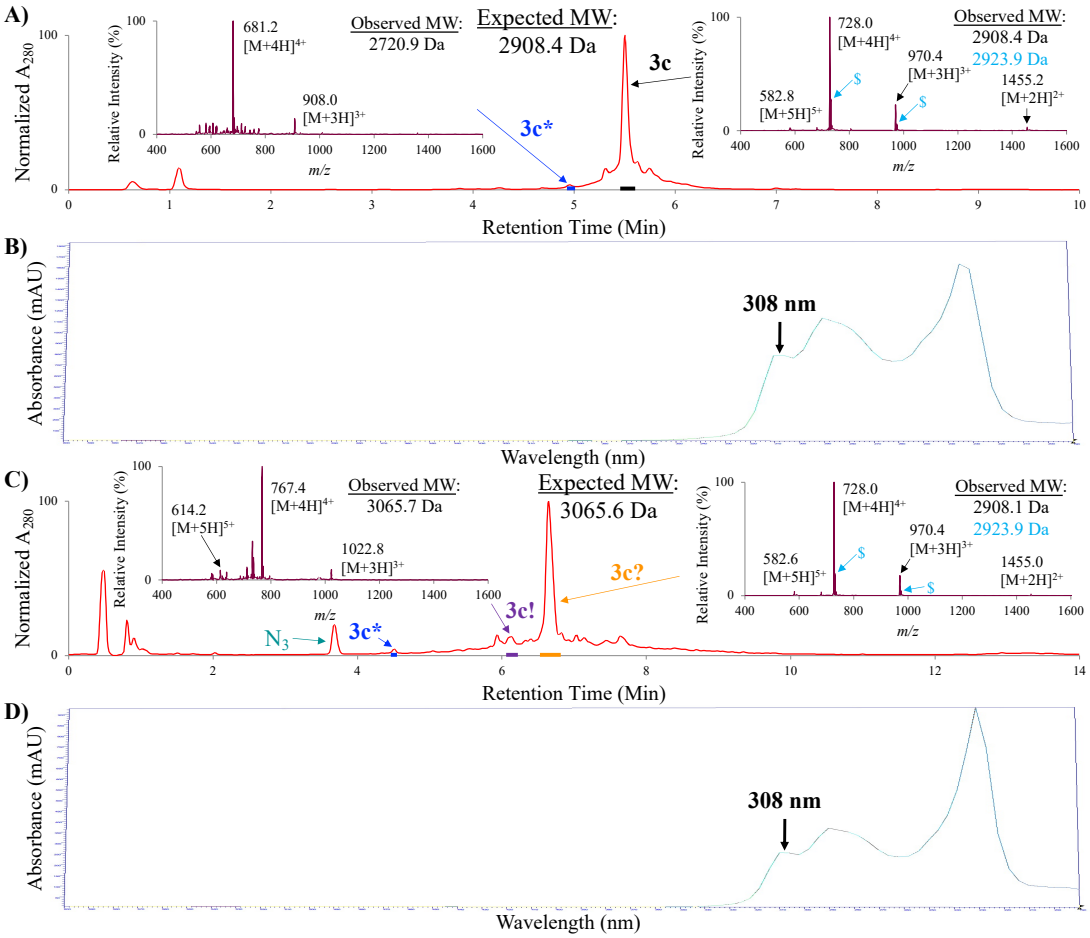


Figure S17: LC/MS analysis of ABCD-Nle(DBCO) (**3c**) cleaved under standard conditions with 50 equiv of $(MeCN)_4CuBF_4$. (A) LC/MS chromatogram of crude **3c**. MS of the major peaks (blue and black bars) are shown. Most of the crude peptide corresponds to **3c**, although the high amount of $(MeCN)_4CuBF_4$ used during cleavage likely causes oxidation (based on the $ ions in the **3c** MS; observed mass of 2923.9 Da). A small amount of degraded DBCO peptide is also observed (**3c***, -188 Da compared to the expected mass). (B) UV absorbance spectrum of the LC/MS chromatogram's largest peak (5.49 – 5.51 min in part A). As expected for DBCO peptides, a local maximum absorbance is observed near 308 nm. This absorbance pattern indicates that most of the DBCO in the **3c** peak is intact. (C) LC/MS chromatogram of the 1 h SPAAC between crude **3c** and 6-azidohexanoic acid. MS of two major peaks (purple and orange bars) are shown. The largest peak corresponds to a peptide containing the expected mass of unreacted **3c**. However, this does not appear to be **3c** that underwent 5-*endo*-*dig*-cycloisomerization of DBCO, as part B does not have the absorbance spectrum of peptides that underwent this rearrangement (Figures S2 and S15). The DBCO on **3c** may still be protected with a copper species due to the large amount of $(MeCN)_4CuBF_4$ used during cleavage (**3c?**). The $ ions in the **3c?** MS are likely **3c** oxidation (observed mass of 2923.9 Da). A small amount of ABCD-Nle(DBCO) with triazole (**3c!**) formed, indicating that some of the **3c** observed in part A was able to undergo SPAAC, despite the large amount of $(MeCN)_4CuBF_4$ used. Degraded DBCO peptide (**3c***, mass of 2720.9 Da is -188 Da compared to the expected mass of **3c**) is still observed. N_3 refers to 6-

azidohexanoic acid. To calculate the amount of reactive DBCO shown in Figure 3(C), the peak area of **3c!** was divided by the total peak area of **3c!**, **3c***, and **3c?**, and the resulting quotient was multiplied by 100. (D) UV absorbance spectrum of the LC/MS chromatogram's largest peak (6.64 – 6.66 min in part C). A local maximum absorbance is detected near 308 nm, indicating that the DBCO in the **3c?** peak is still intact. This absorbance pattern gives more evidence of a copper species still protecting DBCO in aqueous solution, inhibiting SPAAC with 6-azidohexanoic acid. Note that the chromatograms in parts A and C do not have a reference wavelength for baseline correction, based on the potential for rearranged DBCO's absorbance to interfere with this baseline measurement (discussed in Figure S2). This peptide was synthesized via mini scale method B. LC/MS method C was used for parts A and B, while LC/MS method D was used for parts C and D.

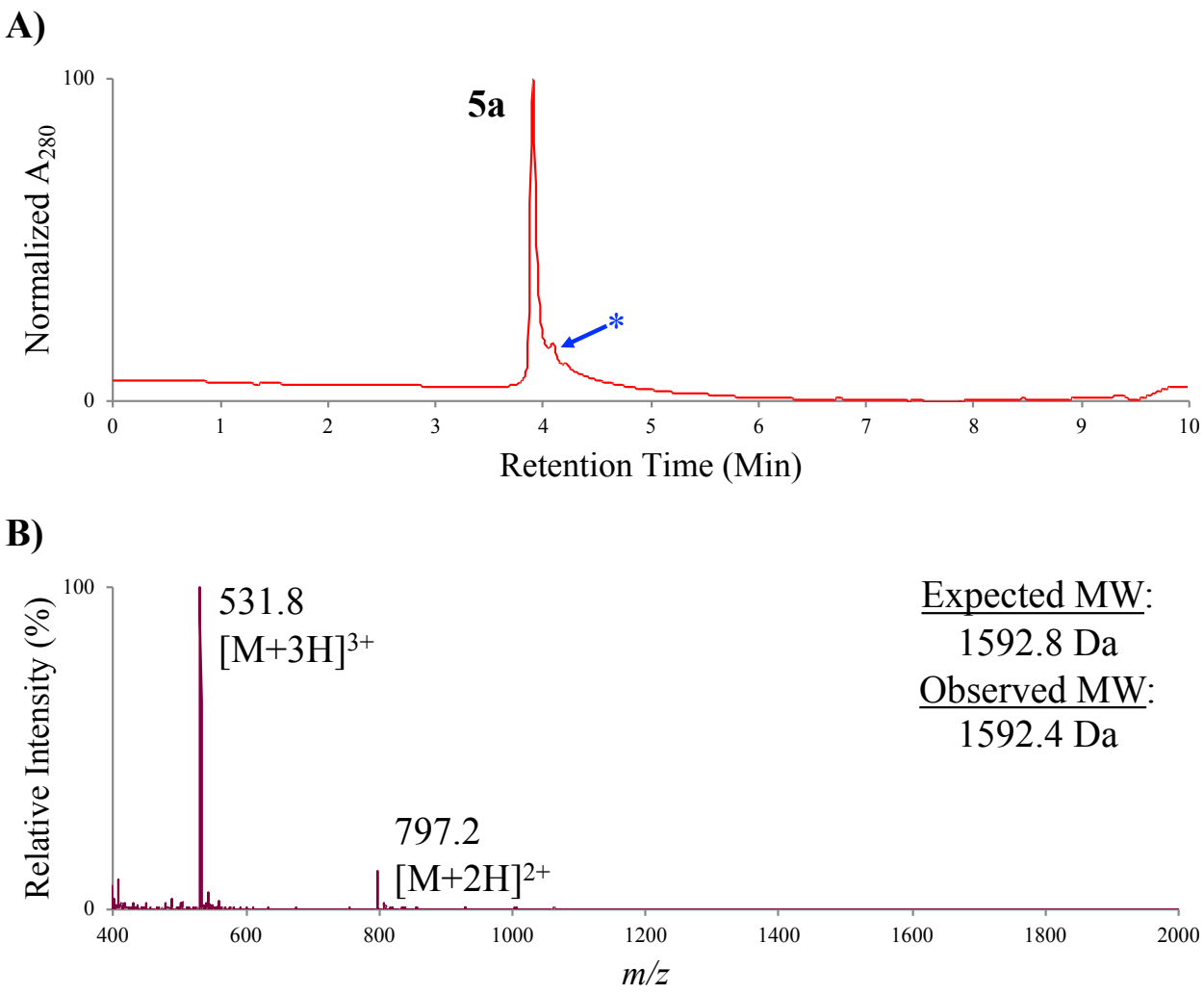


Figure S18: Pure STEVE(Ctrl) peptide (**5a**). (A) LC/MS chromatogram. (B) MS for the entire chromatogram shown in part A (2 – 10 min, as the first 2 min are not injected into the mass spectrometer). * in part A corresponds to a mass of 1508.4 Da, which does not appear within the MS for the entire chromatogram. This minor side product may be **5a** with an uncapped N-terminus and an Arg degraded to ornithine. This peptide was synthesized at 60 µmol scale, cleaved under standard 30 µmol scale conditions, and purified using purification method D. LC/MS method A was used for analysis.

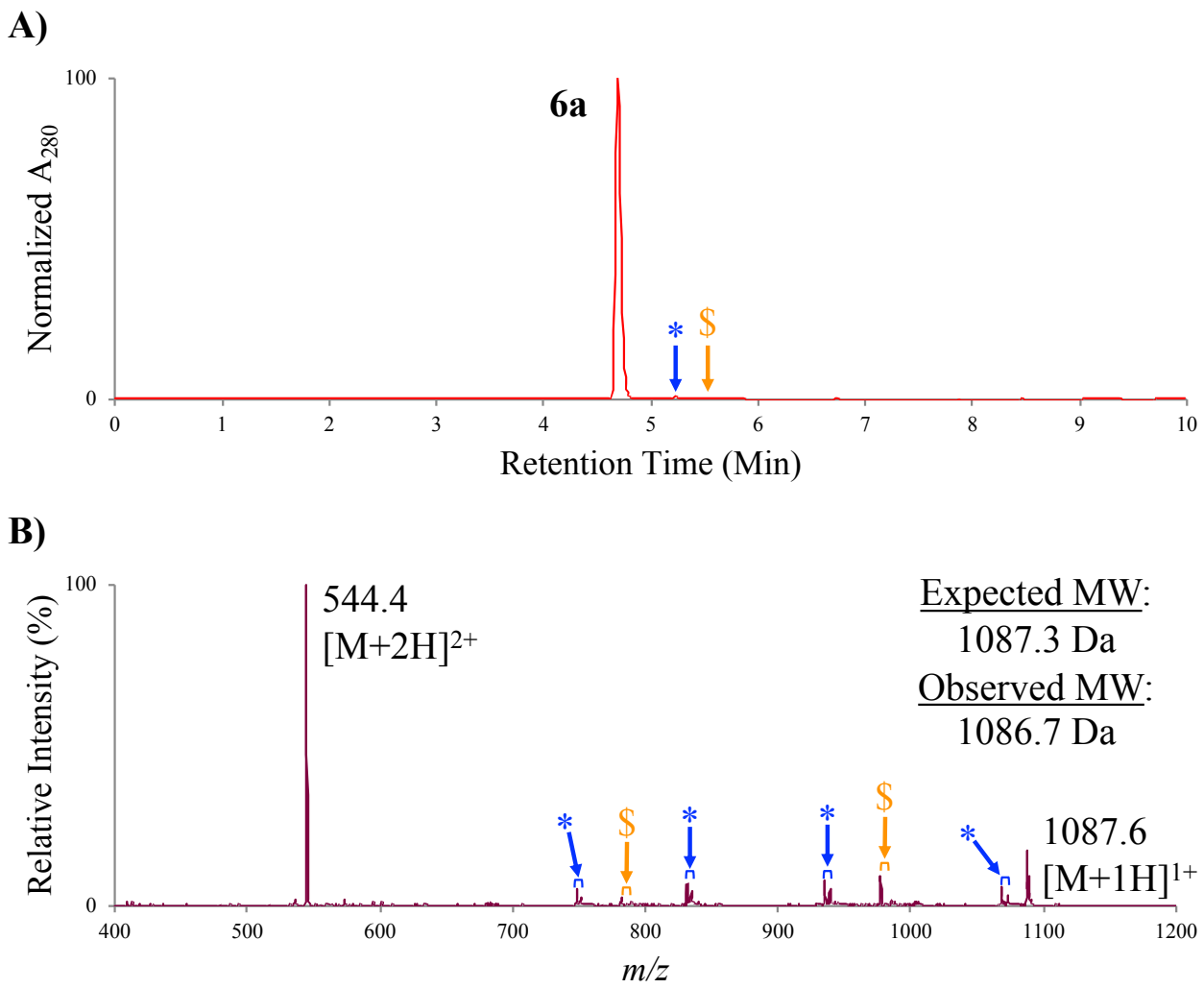


Figure S19: Pure KENT(Ctrl) peptide (**6a**). (A) LC/MS chromatogram. (B) MS for the entire chromatogram shown in part A (2 – 10 min, as the first 2 min are not injected into the mass spectrometer). * and $ correspond to unrelated peptides that were carried over from a previous run on the LC column. This peptide was synthesized at 30 μmol scale, cleaved under standard conditions, and purified using purification method D. LC/MS method A was used for analysis.

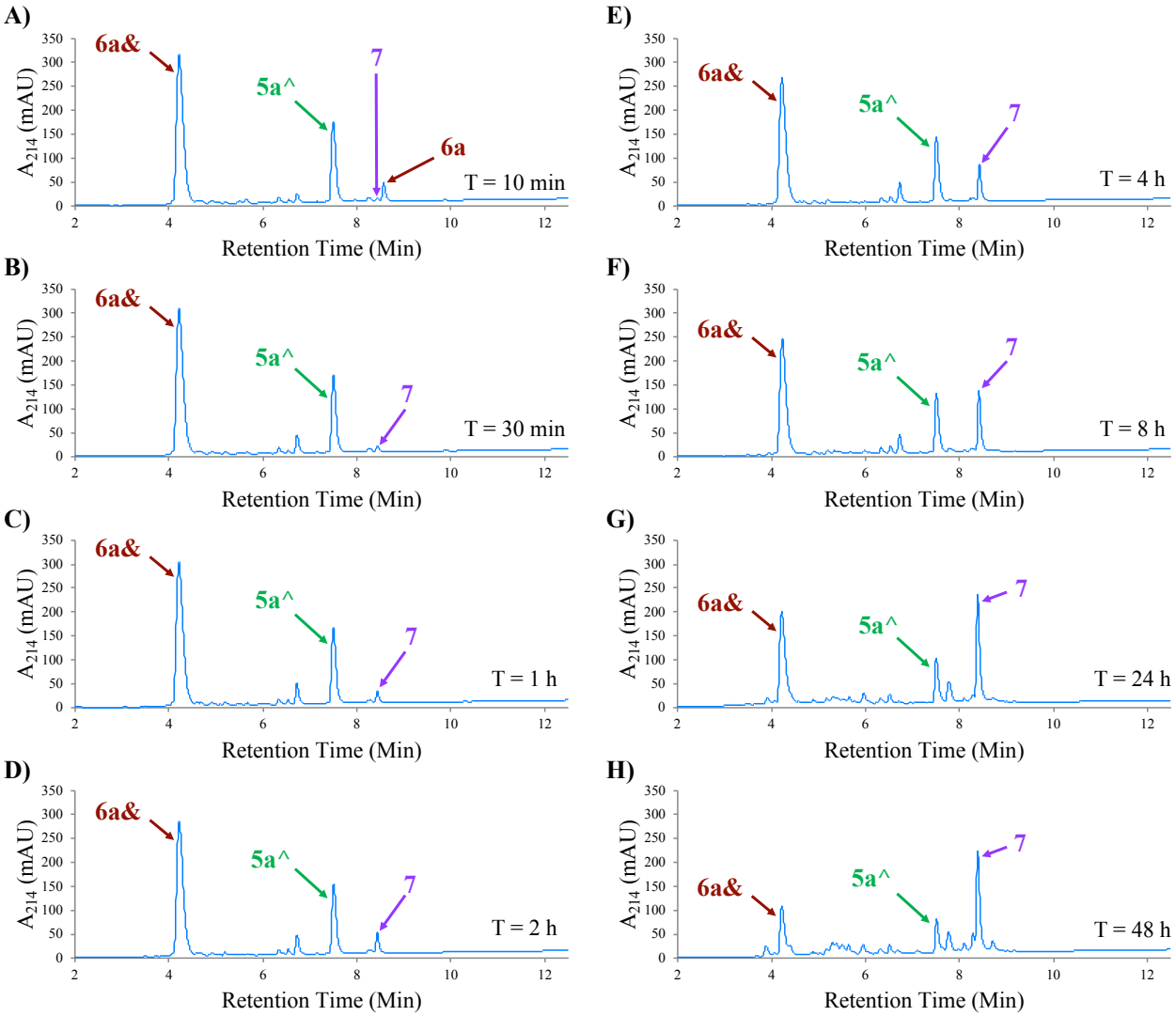


Figure S20: Time-course of the non-templated native chemical ligation between STEVE(Ctrl) (**5a**; 0.4 mM) and KENT(Ctrl) (**6a**; 0.5 mM) peptides (same reaction as time point shown in Figure 4). **6a&** refers to KENT(Ctrl) peptide that does not have S*t*Bu on Cys. **5a^** refers to STEVE(Ctrl) peptide with a C-terminal MTG thioester. **7** refers to the ligated product, full-length STEVEKENT. Note that this is one of two NCL's performed for kinetic calculations and that the traces for the other reaction are not shown. The time points shown here refer to when MTG was added to the reaction. Analytical method B was used for analyses. Scheme S1 shows the detailed reaction conditions for this NCL.

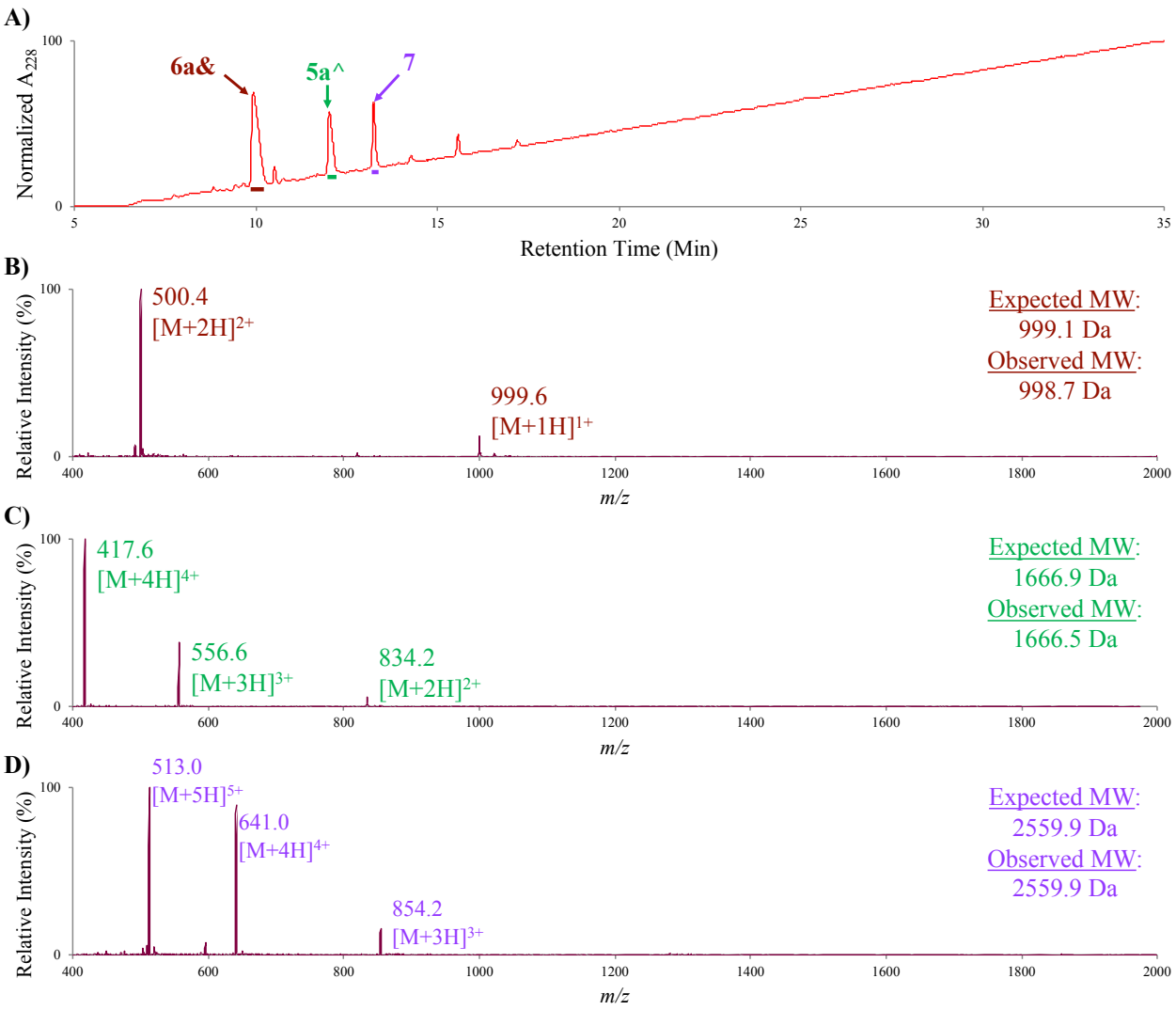


Figure S21: LC/MS analysis of the 8 h time point from the non-templated NCL between STEVE(Ctrl) (**5a**; 0.4 mM) and KENT(Ctrl) (**6a**; 0.5 mM) shown in Figure S20. (A) LC/MS chromatogram (note that the A_{228} increases over time due to acetonitrile being added). (B) MS of the S*t*Bu-deprotected KENT(Ctrl) peptide peak (**6a&**; dark red bar in part A). (C) MS of the STEVE(Ctrl)-MTG peptide peak (**5a^**; green bar in part A). (D) MS of the ligated product peak (**7**; purple bar in part A). LC/MS method E was used for analysis.

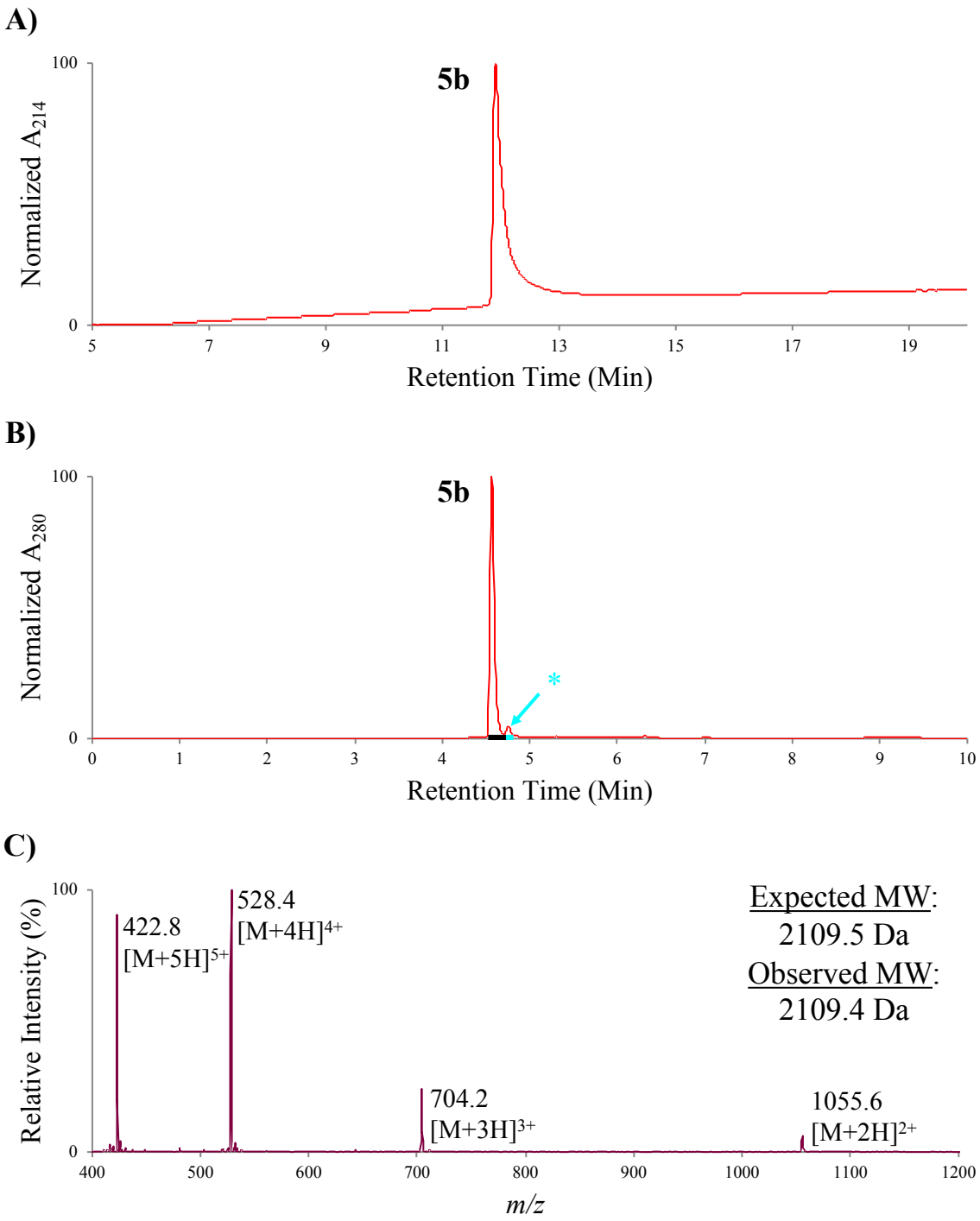


Figure S22: Pure STEVE-Close(CAN) peptide (**5b**). (A) HPLC chromatogram. (B) LC/MS chromatogram. Note that this chromatogram was taken prior to the first SPAAC between **5b** and KENT-Close(CAN) (**6b**), and the peak marked by * (2189.4 Da; cyan bar) formed after lyophilization of pure material. (C) MS of pure **5b** (black bar in part B). This peptide was synthesized at 30 μmol scale, cleaved under standard conditions, and purified using purification methods E and F. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **5b** was 21%.

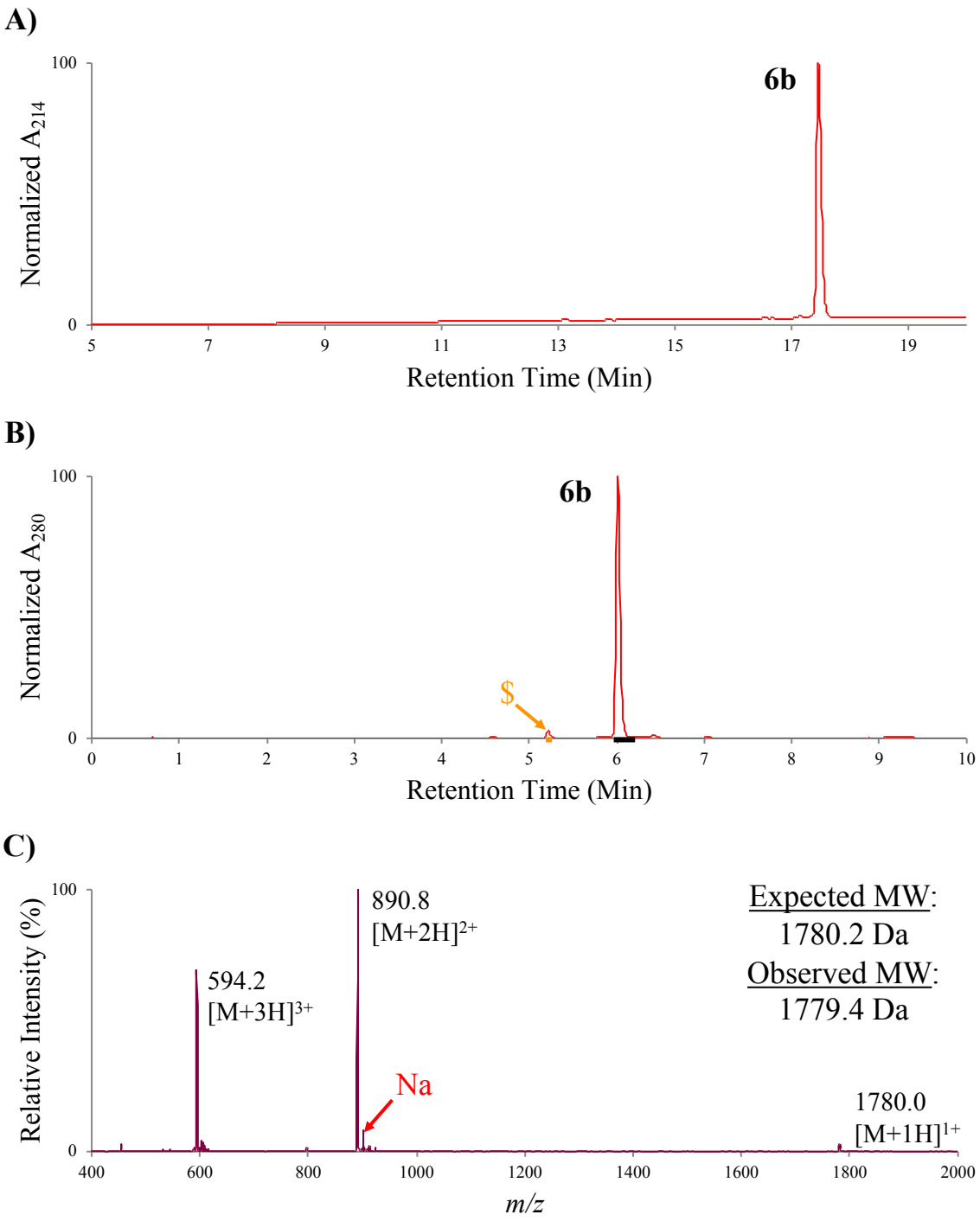


Figure S23: Pure KENT-Close(CAN) peptide (**6b**). (A) HPLC chromatogram. (B) LC/MS chromatogram. Note that this chromatogram was taken prior to the first SPAAC between **6b** and STEVE-Close(CAN) (**5b**), and the peak marked by $ (1592.6 Da; orange bar) represents degraded DBCO as degradation slowly occurs over time. (C) MS of pure **6b** (black bar in part B). Na refers to a sodium adduct (1801.6 Da, +22 Da). This peptide was synthesized at 30 μmol scale, cleaved under standard conditions with 5 equiv of $(MeCN)_4CuBF_4$, and purified using purification methods A, B, and G. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **6b** was 24%.

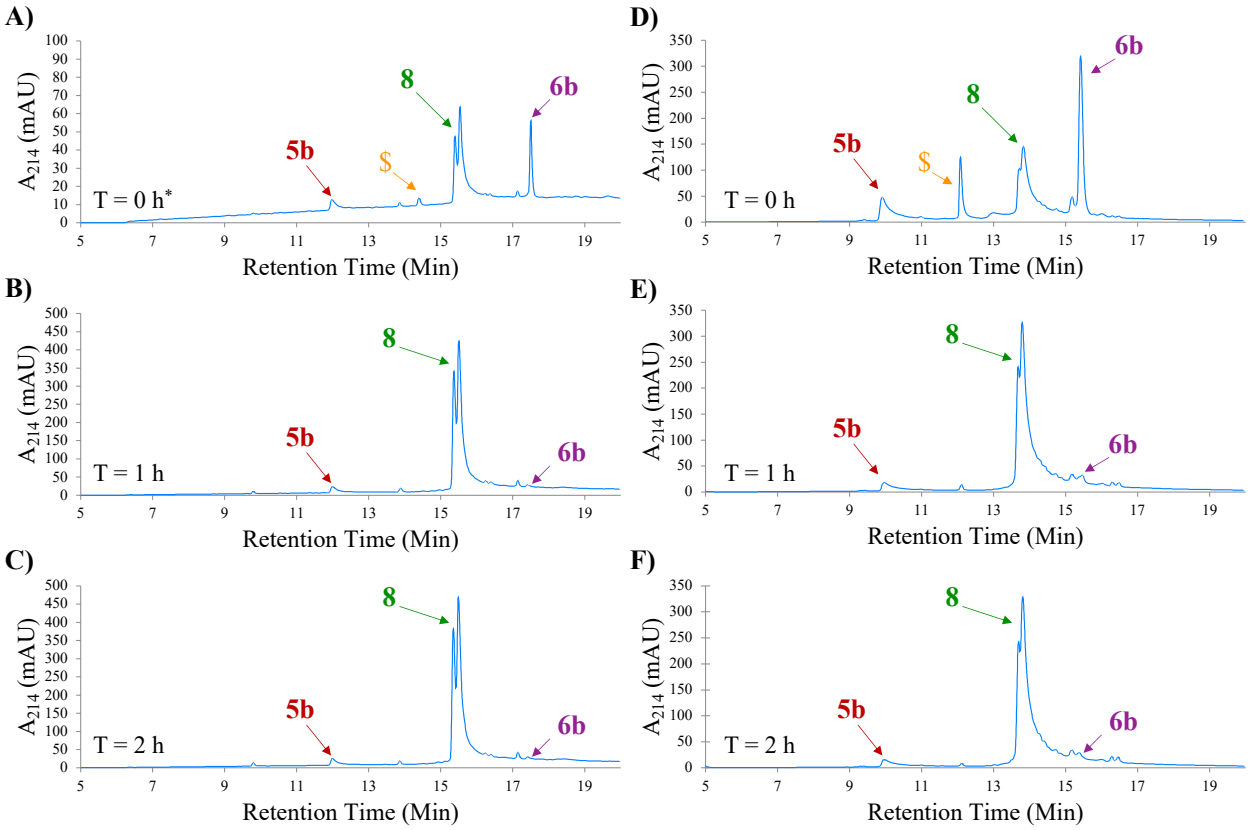


Figure S24: Time-course for the SPAAC between STEVE-Close(CAN) (**5b**) and KENT-Close(CAN) (**6b**). The SPAAC reaction was performed at 1.8 mM **5b** and 1.9 mM **6b** (A-C), as well as 0.4 mM **5b** and 0.5 mM **6b** (D-F). The double peaks of the triazole product (**8**) are expected, as the triazole has two regioisomers. * is to note that the 0 h time point (A) had 1/5 of the injection volume compared to the 1 and 2 h time points (B and C). The peak marked by $ (1592.6 Da) represents degraded DBCO peptide. Since the 1 and 2 h time points looked nearly identical for both the high concentration SPAAC (A-C) and the low concentration SPAAC (D-F), the reactions were taken out of 37°C after 2 h. The product **8** from the high concentration reaction was placed in a -80°C freezer until the CAN reactions were performed. Note that the 0 (A) and 2 h (C) time points are also shown in Figure 5. Analytical method C was used for analyses.

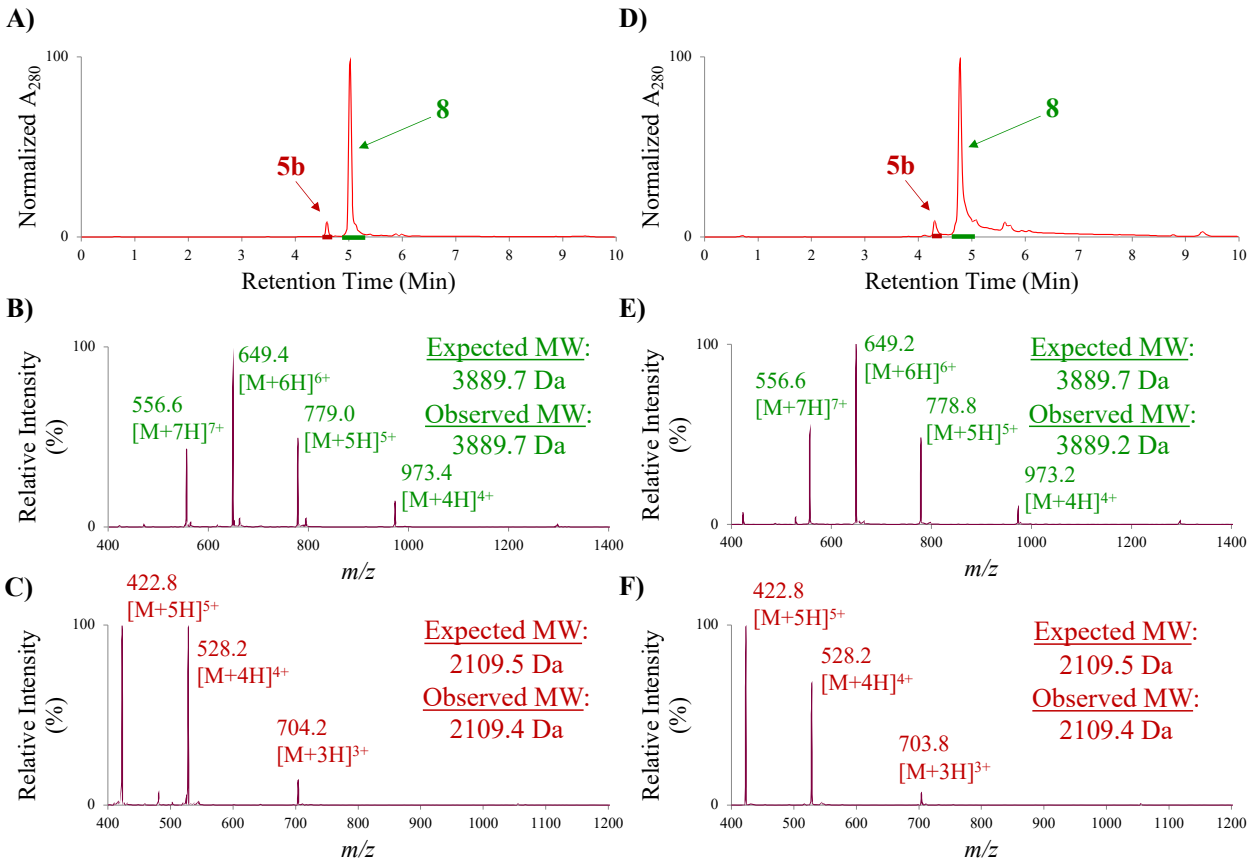


Figure S25: LC/MS analysis of the 2 h time point for the SPAAC reactions between STEVE-Close(CAN) (**5b**) and KENT-Close(CAN) (**6b**) shown in Figure S24 at high peptide concentration (A-C) and at low peptide concentration (D-E). (A and D) LC/MS chromatograms. (B and E) MS of the triazole peptide peak (**8**; green bars in parts A and D). (C and F) MS of the unreacted **5b** peak (dark red bars in parts A and D). LC/MS method C was used for analyses**.**

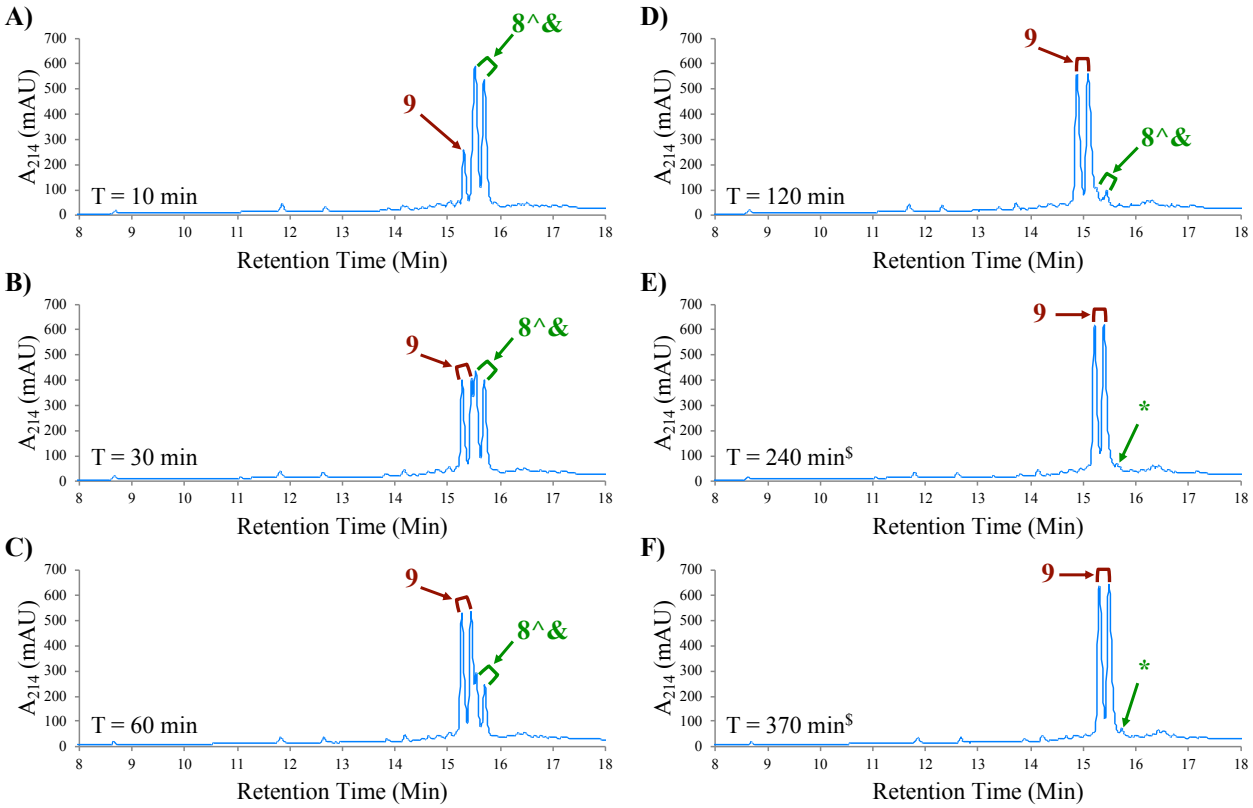


Figure S26: Time-course for the initial click-assisted native chemical ligation of 0.5 mM STEVEKENT-Close(CAN) Post-Click peptide (**8** not isolated after the high concentration SPAAC shown in Figures S24-25). **8^&** refers to S*t*Bu-deprotected **8** that contains a C-terminal MTG thioester. **9** refers to the ligated product. The double peaks of the peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that (E) is also shown in Figure 5. The $ in (E) and (F) are to note that the retention time of **9** shifted to the right due to backpressure issues on the HPLC instrument. As a result, this reaction was not used for kinetic analysis of the CAN reaction (see Figures S31-32). The * in (E) and (F) are to note that the peak shown is not **8^&**, but rather a side-product that elutes near **8^&** (based on this peak not changing in (F) and no unligated material being detected by mass spectrometry). Since the 240 min and 370 min time points are nearly identical, the CAN reaction was finished after 4 h. After the 370 min time point was taken, the reaction was placed in a -80°C freezer until the Ddap linker cleavage reaction was performed. Analytical method C was used for analyses.

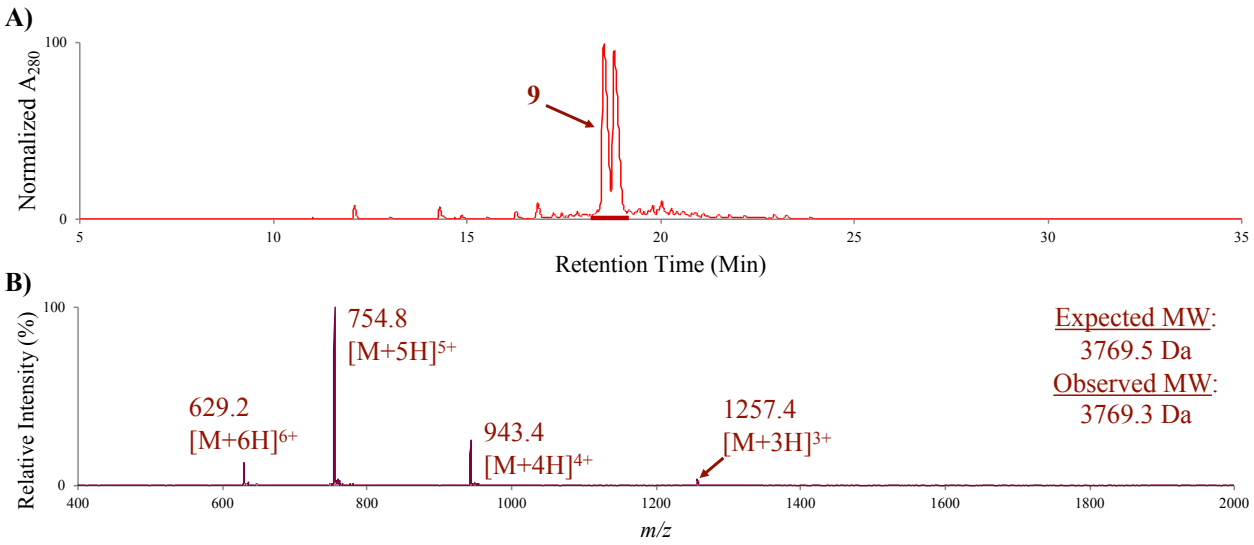


Figure S27: LC/MS analysis of the 370 min time point from the initial click-assisted native chemical ligation of STEVEKENT-Close(CAN) Post-Click peptide (**8**; 0.5 mM) shown in Figure S26. (A) LC/MS chromatogram. The double peaks of the ligated product (**9**) are expected, as the triazole has two regioisomers. (B) MS of **9** (dark red bar in part A). LC/MS method F was used for analysis.

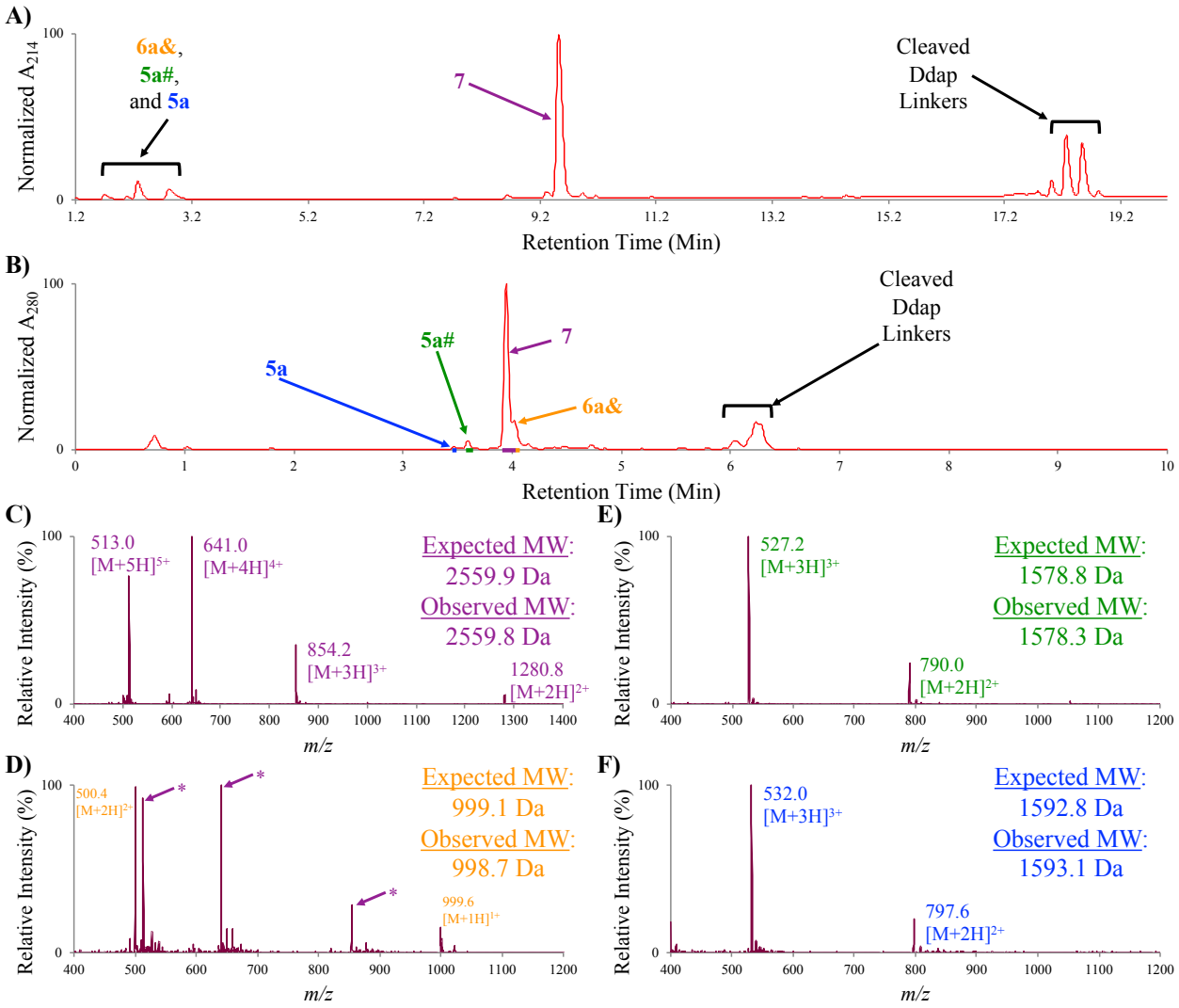


Figure S28: Analysis of the Ddap linker removal reaction performed on the STEVEKENT-Close(CAN) Post-NCL peptide (**9** not isolated after the CAN shown in Figures S26-27). Traceless removal of the Ddap linkers was achieved by treating **9** with ~1 M hydroxylamine for 2 h at rt (see Scheme S2). The reaction was then quenched via 1:1 dilution with 20% acetic acid in LC/MS-grade water. (A) HPLC chromatogram of the quenched linker removal reaction. Note that this chromatogram is also shown in Figure 5, but the retention time range is extended here to show the unligated STEVE and KENT peptides that elute before the gradient starts at 5 min. **6a&** refers to S*t*Bu-deprotected KENT peptide. **5a#** refers to STEVE peptide that underwent thioester hydrolysis during CAN. **5a** corresponds to STEVE peptide that did not get activated to form an acyl azide prior to the CAN reaction, causing the C-terminal hydrazide to remain. **7** refers to the full-length STEVEKENT peptide. (B) LC/MS chromatogram of the quenched linker removal reaction. (C) MS of **7** (purple bar in part B). (D) MS of **6a&** (orange bar in part B). * refers to **7**, as **6a&** elutes closely to full-length STEVEKENT. (E) MS of **5a#** (green bar in part B). (F) MS of **5a** (blue bar in part B). Analytical method C and LC/MS method C were used for analyses.

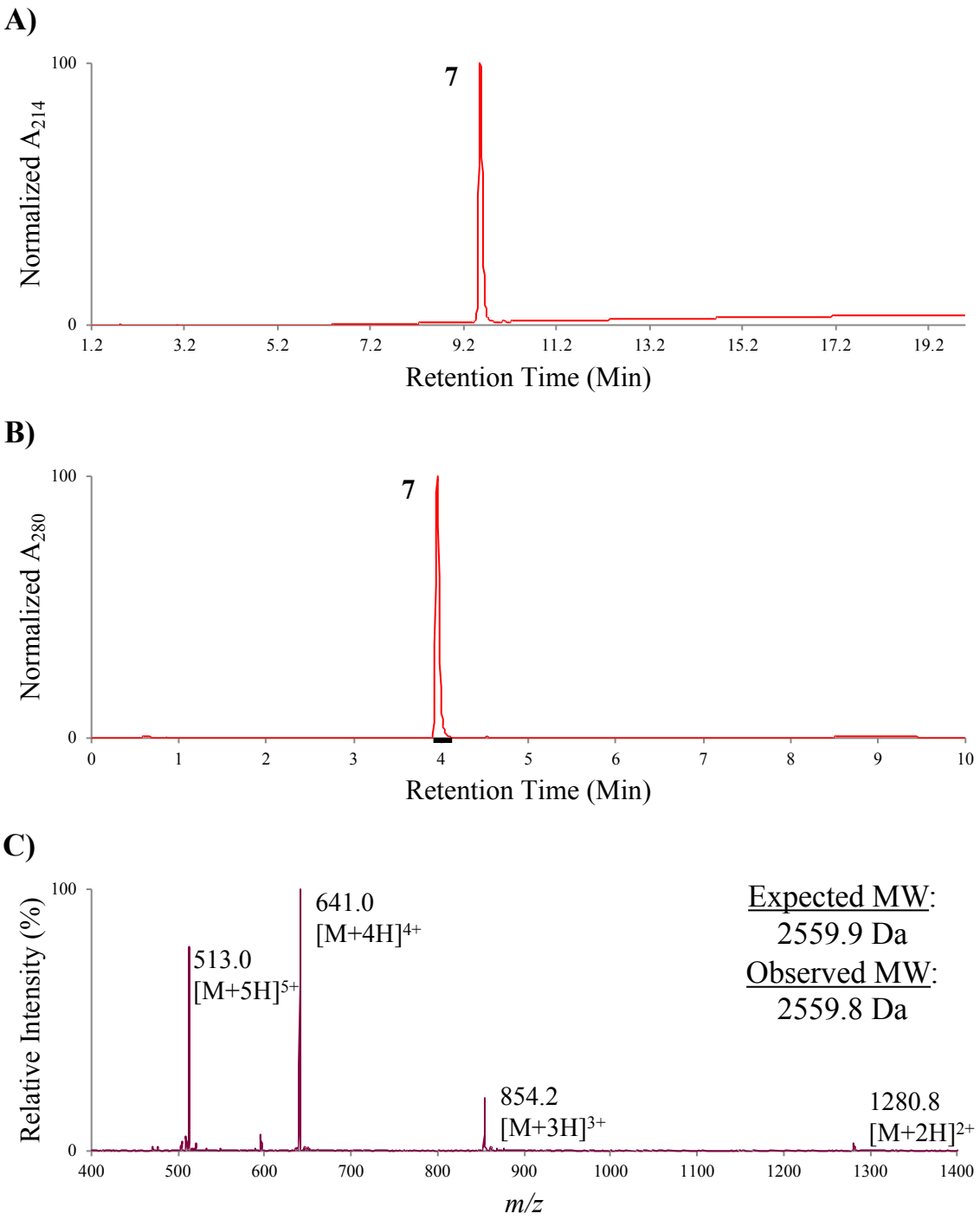


Figure S29: Purified STEVEKENT peptide (**7**) after one-pot SPAAC, CAN, and Ddap linker removal (Figures S24-28). (A) HPLC chromatogram. Note that this chromatogram is also shown in Figure 5, but the retention time range is extended here to match the time range of Figure S28(A). (B) LC/MS chromatogram. (C) MS of pure **7** (black bar in part B). Note that the deconvoluted MS shown in Figure 5 was generated with the UniDec software.[7] This peptide was purified using purification method H. Analytical method C and LC/MS method C were used for analyses.

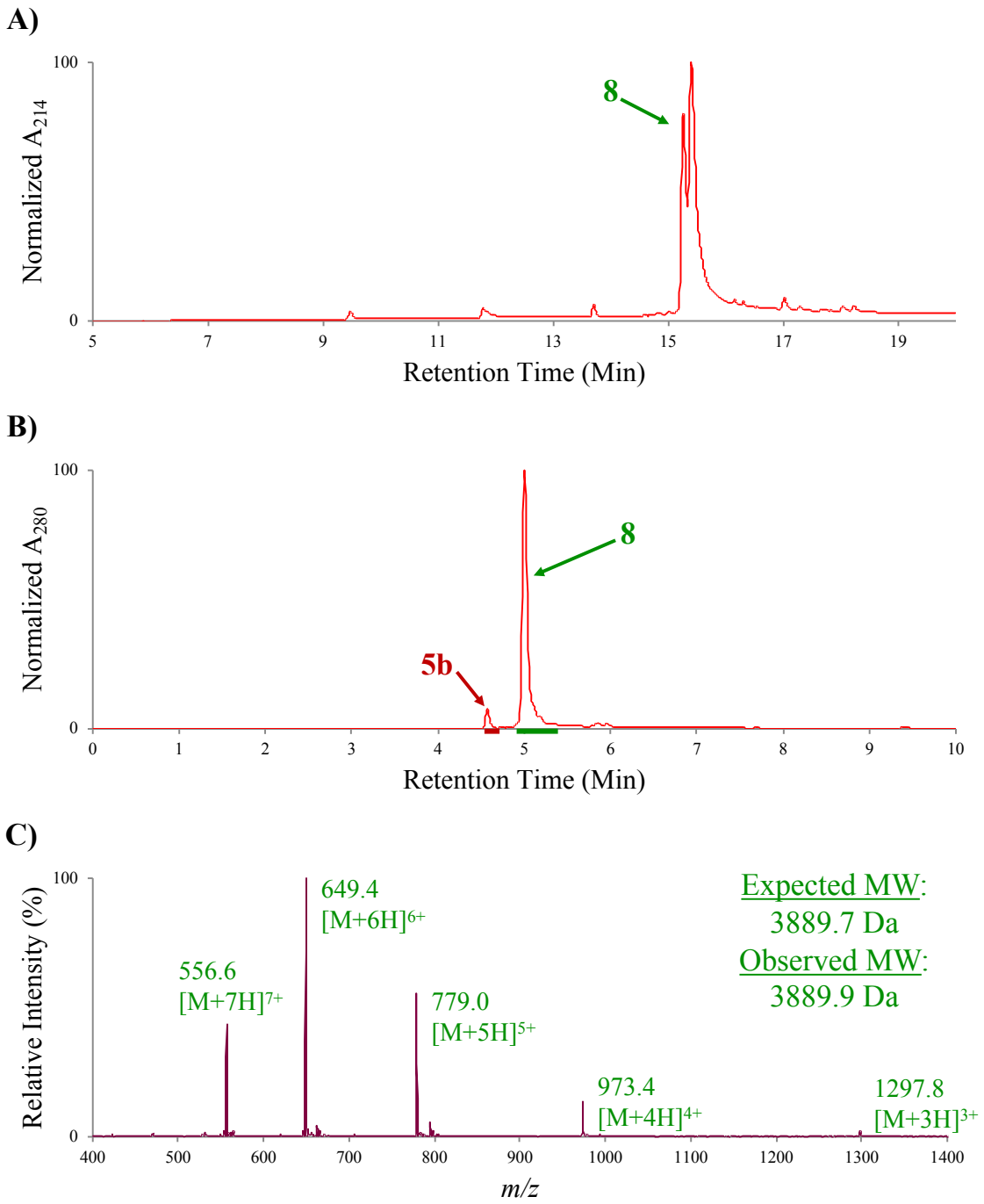


Figure S30: Analysis of the 2 h time point from the second SPAAC between STEVE-Close(CAN) (**5b**) and KENT-Close(CAN) (**6b**). This SPAAC was performed via the same conditions as the first high concentration SPAAC (Figures S24-25), and this reaction was needed to prepare more triazole peptide (**8**) for kinetic analysis of the STEVEKENT CAN. (A) HPLC chromatogram. As expected, this chromatogram is similar to the 2 h time point of the first SPAAC (Figure S24(C)). (B) LC/MS chromatogram. As expected, this chromatogram is similar to the 2 h time point of the first SPAAC (Figure S25(A)), and some unreacted **5b** (observed mass of 2109.2 Da) is also present here. (C) MS of **8** (green bar in part B). Analytical method C and LC/MS method C were used for analyses.

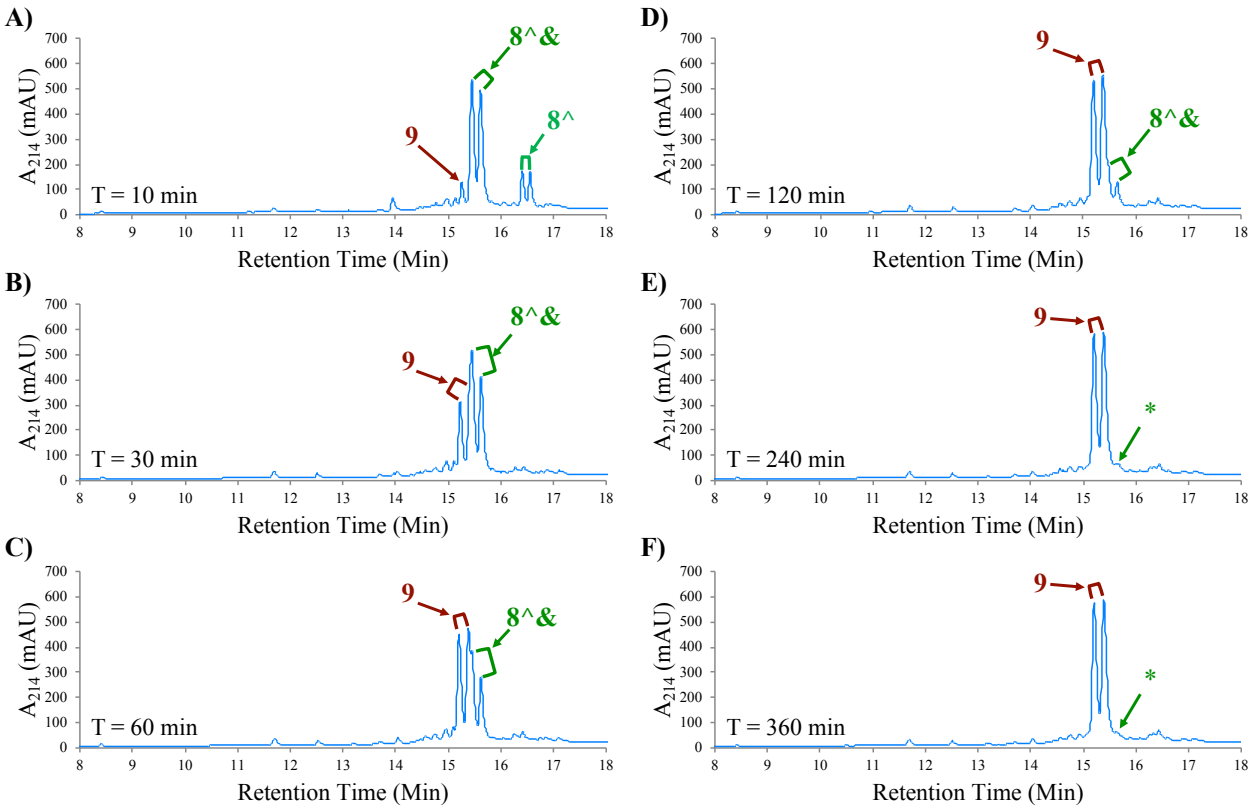


Figure S31: Time-course for one of the click-assisted native chemical ligations of 0.5 mM STEVEKENT-Close(CAN) Post-Click peptide (**8** not isolated after the SPAAC shown in Figure S30) used to calculate kinetic rates. **8^&** refers to S*t*Bu-deprotected **8** that contains a C-terminal MTG thioester. **8^** corresponds to S*t*Bu-protected **8** that contains a C-terminal MTG thioester. **9** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that **8^** was detected in (A) for both CAN reactions used for determining kinetic rates. Since this time point was taken immediately after adding TCEP to the reaction, not all of the S*t*Bu has been removed yet. Due to the HPLC backpressure issue that occurred during the initial CAN of **8** (Figure S26), S*t*Bu-protected peptide is not observed since the time point set at rt long enough for S*t*Bu removal to occur prior to HPLC injection. The * in (E) and (F) are to note that the peak shown is not **8^&**, but rather a side-product that elutes near **8^&** (based on this peak not changing in (F) and no unligated material being detected by mass spectrometry). Note that the time points from the second CAN of **8** used for determining the kinetic rate are not shown. Since a regioisomer from both **8^&** and **9** nearly co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **8^&** and the left peak for **9**). In addition, only the right peak area of **8^** was used to calculate the total area of unligated peptide in the 10 min time points. Analytical method C was used for analyses.

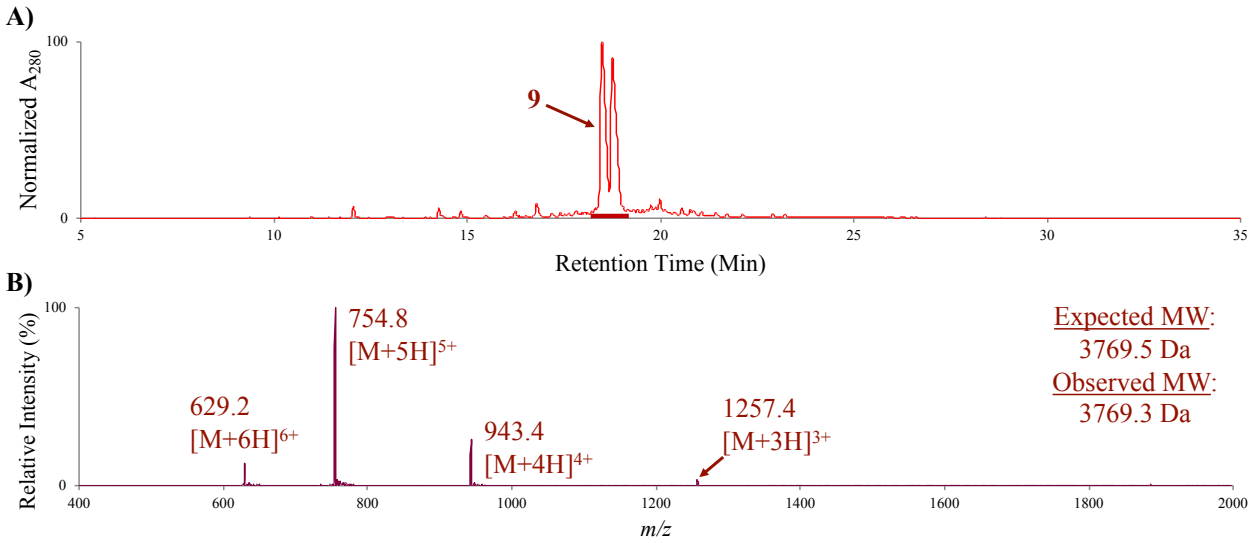


Figure S32: LC/MS analysis of the 360 min time point from the click-assisted native chemical ligation of STEVEKENT-Close(CAN) Post-Click peptide (**8**; 0.5 mM) shown in Figure S31. (A) LC/MS chromatogram. The double peaks of the ligated product (**9**) are expected, as the triazole has two regioisomers. (B) MS of **9** (dark red bar in part A). LC/MS method F was used for analysis.

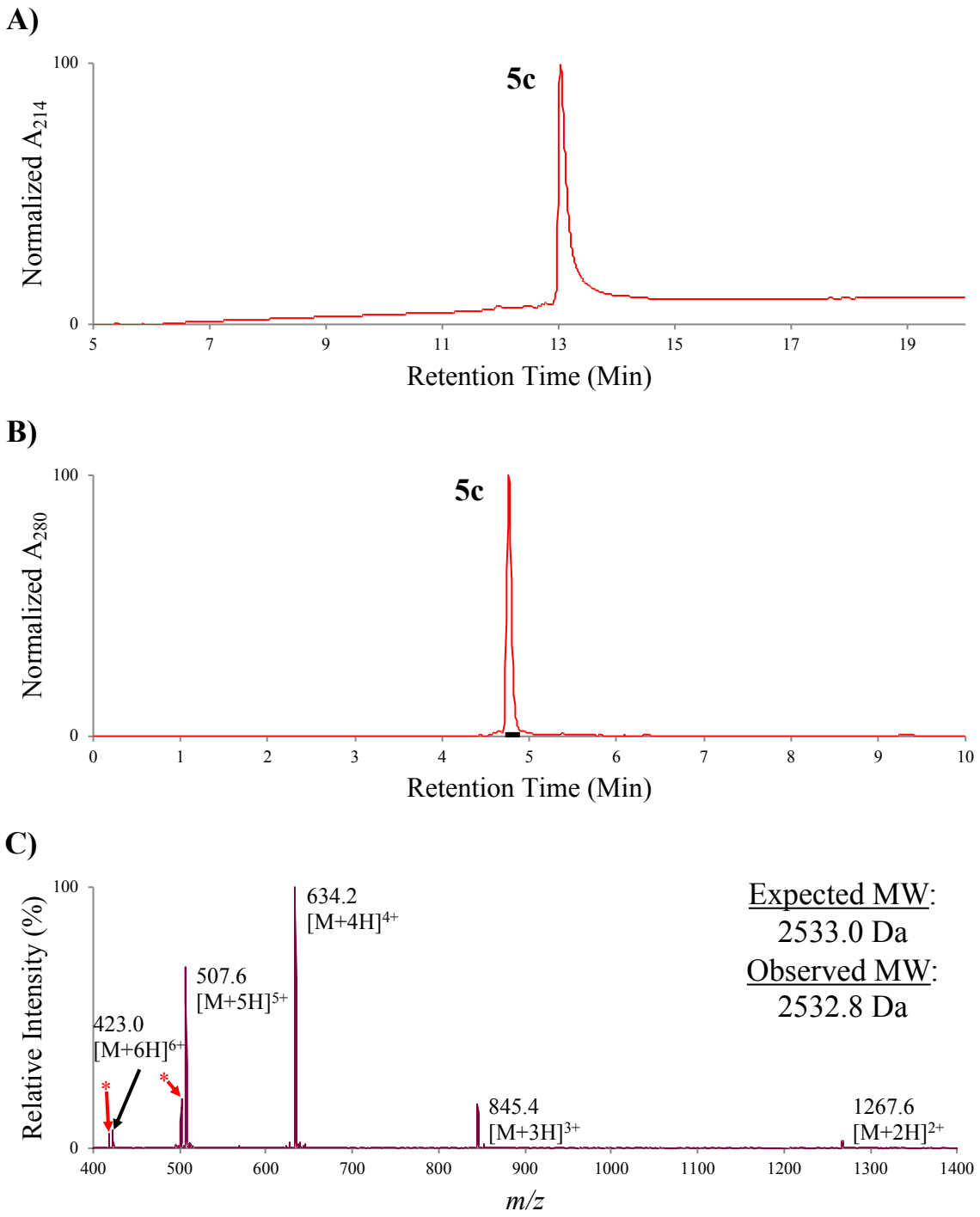


Figure S33: Pure STEVE-Far(CAN) peptide (**5c**). (A) HPLC chromatogram. (B) LC/MS chromatogram. Note that this chromatogram was taken prior to SPAAC between **5c** and KENT-Far(CAN) (**6c**). (C) MS of pure **5c** (black bar in part B). * corresponds to an observed mass of 2504.7 Da, which is likely **5c** with reduced azide. This peptide was synthesized at 30 µmol scale, cleaved under standard conditions, and purified using purification method F. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **5c** was 21%.

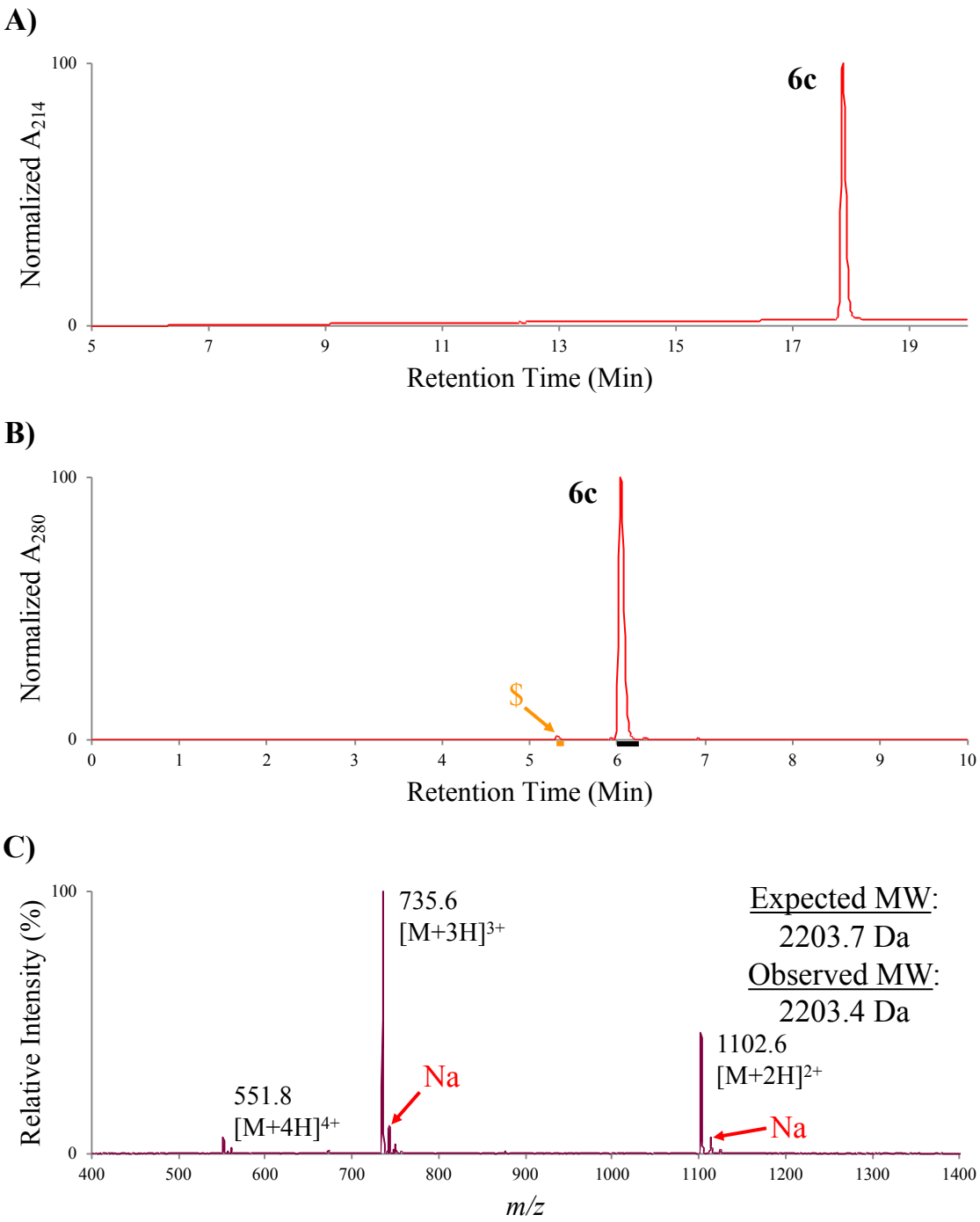


Figure S34: Pure KENT-Far(CAN) peptide (**6c**). (A) HPLC chromatogram. (B) LC/MS chromatogram. Note that this chromatogram was taken prior to SPAAC between **6c** and STEVE-Far(CAN) (**5c**). The peak marked by $ (observed mass of 2015.9 Da) represents degraded DBCO peptide, as degradation slowly occurs over time. (C) MS of pure **6c** (black bar in part B). Na refers to a sodium adduct on **6c** (observed mass of 2225.1 Da). This peptide was synthesized at 30 μmol scale, cleaved under standard conditions with 5 equiv of $(MeCN)_4CuBF_4$, and purified using purification methods I and J. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **6c** was 16%.

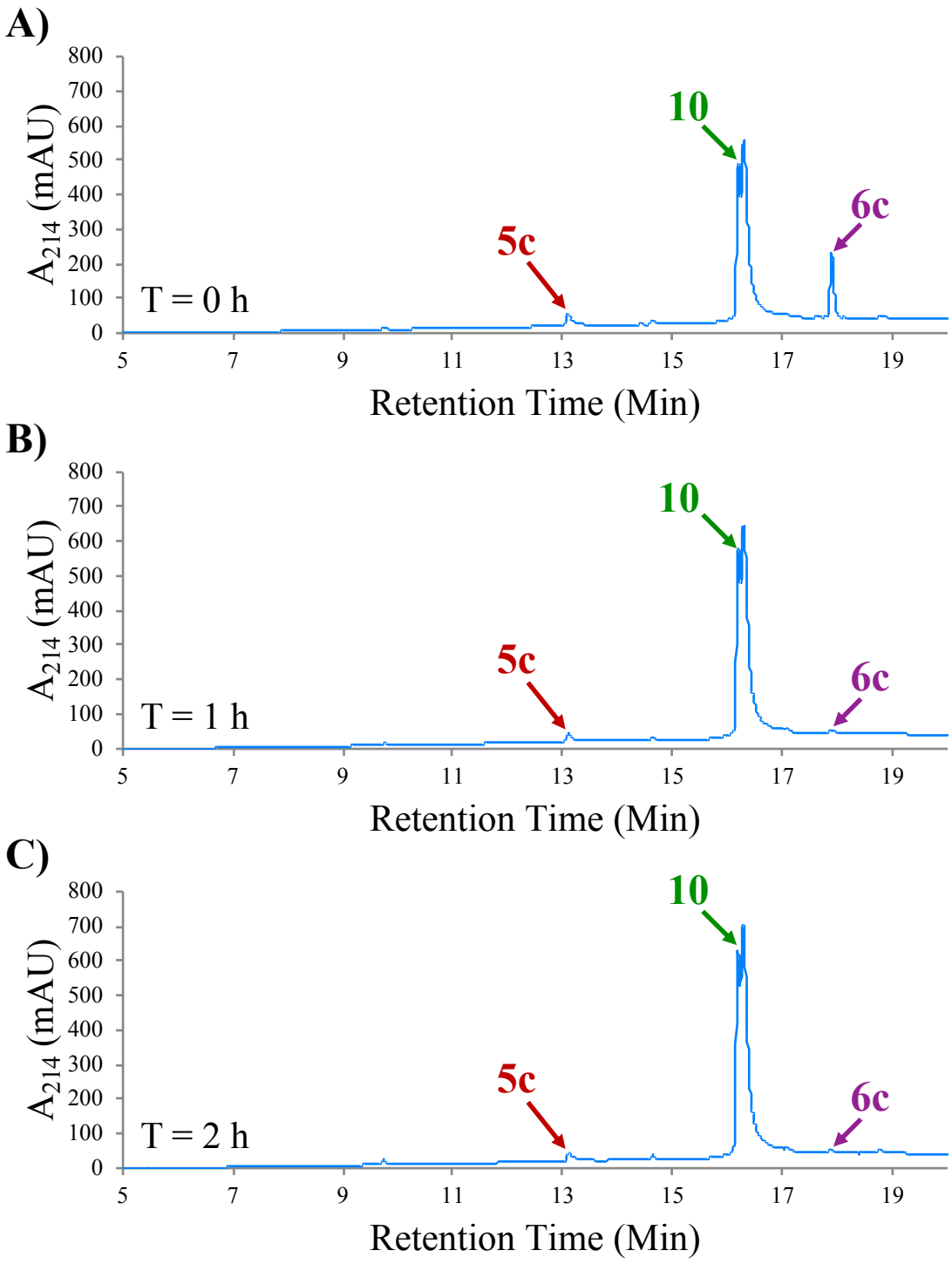


Figure S35: Time-course for the SPAAC between STEVE-Far(CAN) (**5c**; 2.5 mM) and KENT-Far(CAN) (**6c**; 2.6 mM). The double peaks of the triazole product (**10**) are expected, as triazole has two regioisomers. Since **6c** was not observed in the 2 h time point, the completed SPAAC reaction was taken out of 37°C after 2 h and placed in -80°C until the CAN reactions were performed. Analytical method C was used for analyses.

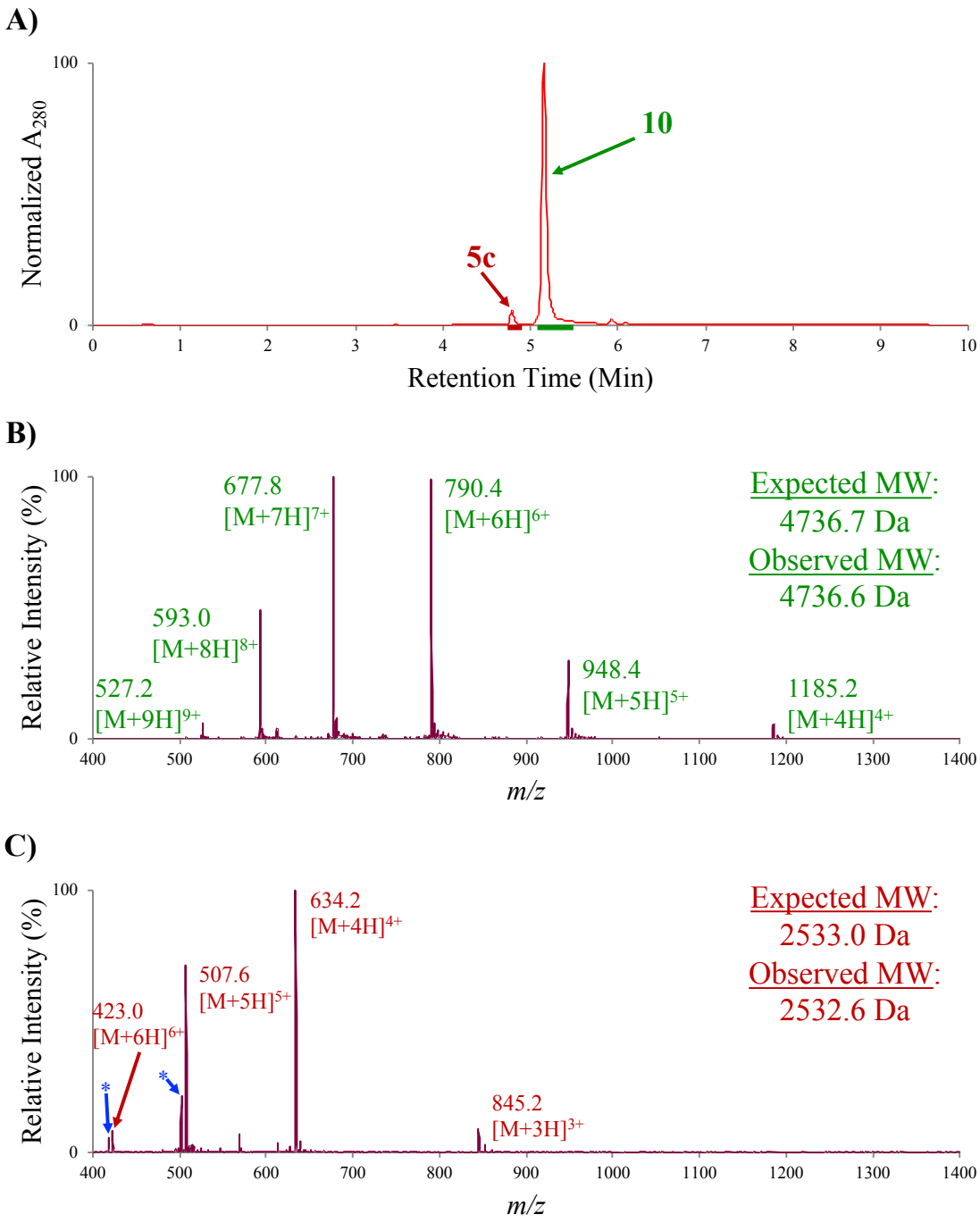


Figure S36: LC/MS analysis of the 2 h time point for the SPAAC between STEVE-Far(CAN) (**5c**) and KENT-Far(CAN) (**6c**) shown in Figure S35. (A) LC/MS chromatogram. (B) MS of the triazole peptide peak (**10**; green bar in part A). (C) MS of the unreacted **5c** (dark red bar in part A). * corresponds to an observed mass of 2504.7 Da, which is likely **5c** with reduced azide. LC/MS method C was used for analysis.

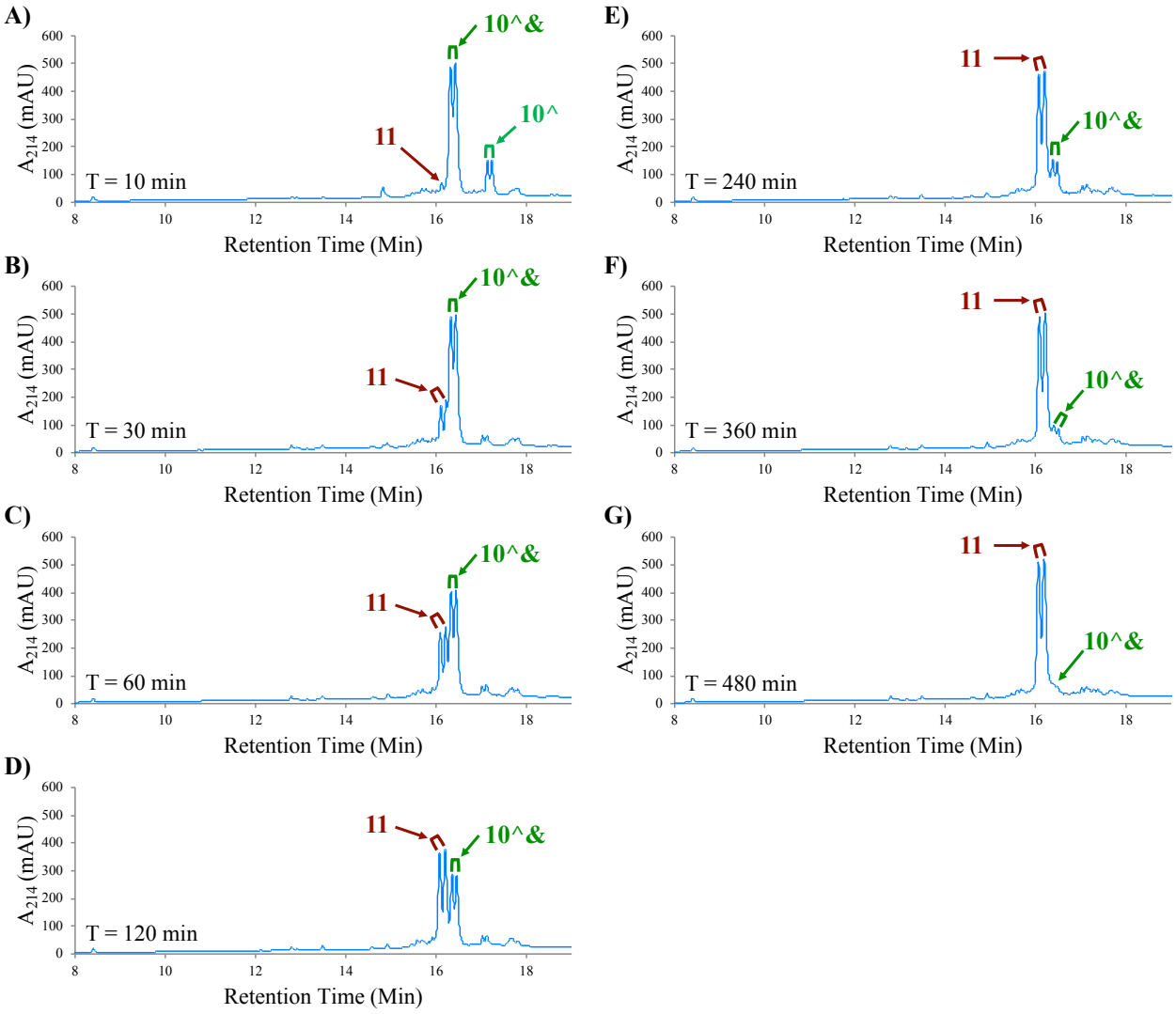


Figure S37: Time-course for one of the click-assisted native chemical ligations of 0.5 mM STEVEKENT-Far(CAN) Post-Click peptide (**10** not isolated after the SPAAC shown in Figures S35-36). **10^&** refers to S*t*Bu-deprotected **10** that contains a C-terminal MTG thioester. **10^** corresponds to S*t*Bu-protected **10** that contains a C-terminal MTG thioester. **11** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that **10^** was detected in (A) for both CAN reactions performed to determine kinetic rates. Since this time point was taken immediately after adding TCEP to the reaction, not all of the S*t*Bu has been removed yet. The reaction was finished after 480 min, as nearly all of **10^&** reacted. Note that the time points from the second CAN of **10** used for determining the kinetic rate are not shown. Since a regioisomer from both **10^&** and **11** nearly co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **10^&** and the left peak for **11**). In addition, only the right peak area of **10^** was used to calculate the total area of unligated peptide in the 10 min time points. Analytical method C was used for analyses.

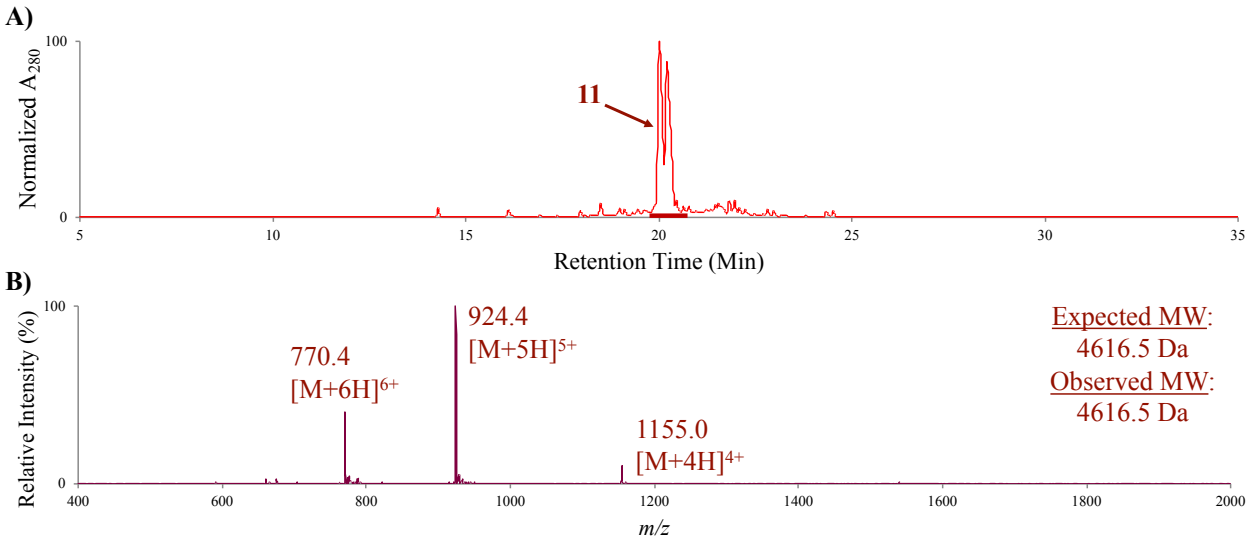


Figure S38: LC/MS analysis of the 480 min time point from the click-assisted native chemical ligation of 0.5 mM STEVEKENT-Far(CAN) Post-Click peptide (**10**) shown in Figure S37. (A) LC/MS chromatogram. The double peaks of the ligated product (**11**) are expected, as the triazole has two regioisomers. (B) MS of **11** (dark red line in part A). LC/MS method F was used for analysis.

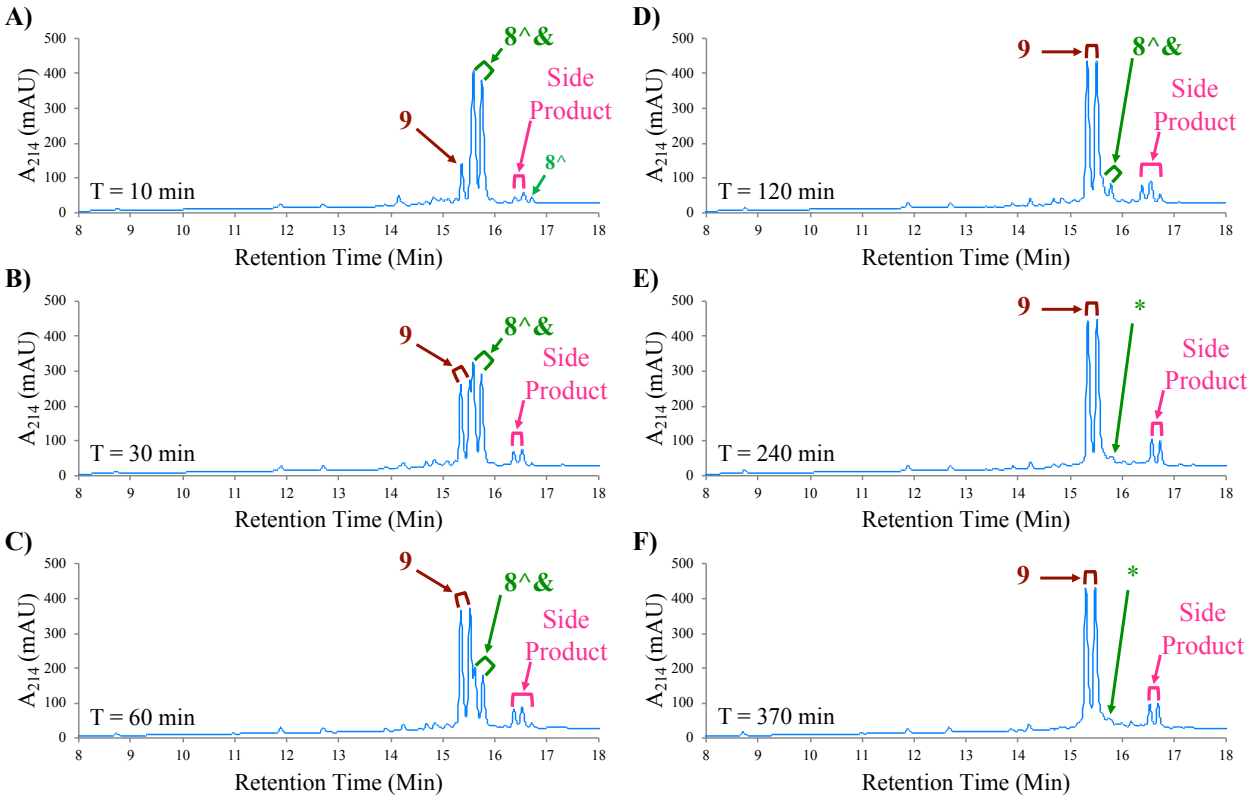


Figure S39: Time-course for the click-assisted native chemical ligation of 0.05 mM STEVEKENT-Close(CAN) Post-Click peptide (**8** not isolated after the high concentration SPAAC shown in Figures S24-25), performed under the same conditions as the 0.5 mM CAN reaction (100 mM MTG and 100 mM TCEP). **8^&** refers to S*t*Bu-deprotected **8** that contains a C-terminal MTG thioester. **8^** corresponds to S*t*Bu-protected **8** that contains a C-terminal MTG thioester. **9** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. The * in (E) and (F) are to note that the peak shown is not **8^&**, but rather a side-product that elutes near **8^&** (based on this peak not changing in (F) and no unligated material being detected by mass spectrometry). Although this reaction formed the desired ligated peptide (**9**), a decent amount of an undesired side-product also formed. Note that the side-product originally has 2 peaks (10 min and 30 min), then forms three peaks (60 min and 120 min), and then goes back to 2 peaks that have an increased retention time compared to the original 2 peaks (240 min and 370 min). Initially, the side-product appears to result from the N-terminal Cys being capped in **8^&**, as mass spectrometry indicates this initial side-product has an observed mass of +74 Da compared to **8^&** (Figure S40(C)). This mass suggests that capping occurred via acylation from an MTG acyl donor. Over time, the capped **8^&** side-product reacts further, producing an observed mass of +146 Da compared to **8^&**. This may be from the initial side-product undergoing a second acylation from an MTG acyl donor. Analytical method C was used for analyses.

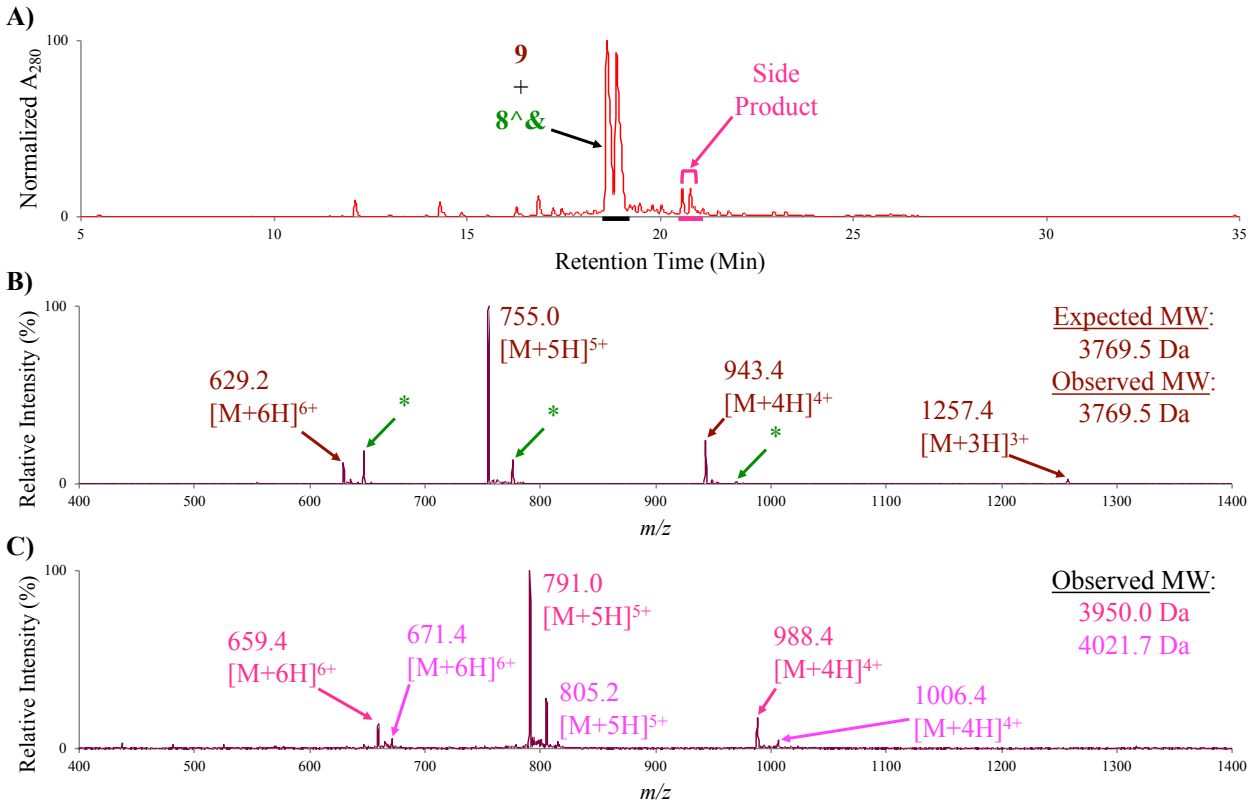


Figure S40: LC/MS analysis of the 60 min time point from the click-assisted native chemical ligation of STEVEKENT-Close(CAN) Post-Click peptide (**8**) shown in Figure S39 (0.05 mM **8**, 100 mM MTG, and 100 mM TCEP). (A) LC/MS chromatogram. **8^&** refers to S*t*Bu-deprotected **8** that contains a C-terminal MTG thioester. **9** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. (B) MS of the **9**/**8^&** peptide peaks (black bar in part A). The expected mass refers to **9**. The ions marked with * refer to **8^&** (observed mass of 3876.0 Da). (C) MS of the side-product peaks (pink bar in part A). The majority of the side-product corresponds to an observed mass that is +74 Da compared to **8^&** (3950.0 Da), which is likely **8^&** that underwent acylation from an MTG acyl donor. An observed mass that is +146 Da compared to **8^&** is also present (4021.7 Da). LC/MS analysis of the 370 min time point (not shown) revealed that the side-product almost completely converts to the +146 Da mass, indicating that the initial side-product (+74 Da) continues to react over time, and the increase in mass suggests that a second acylation may occur via an MTG acyl donor. LC/MS method F was used for analysis.

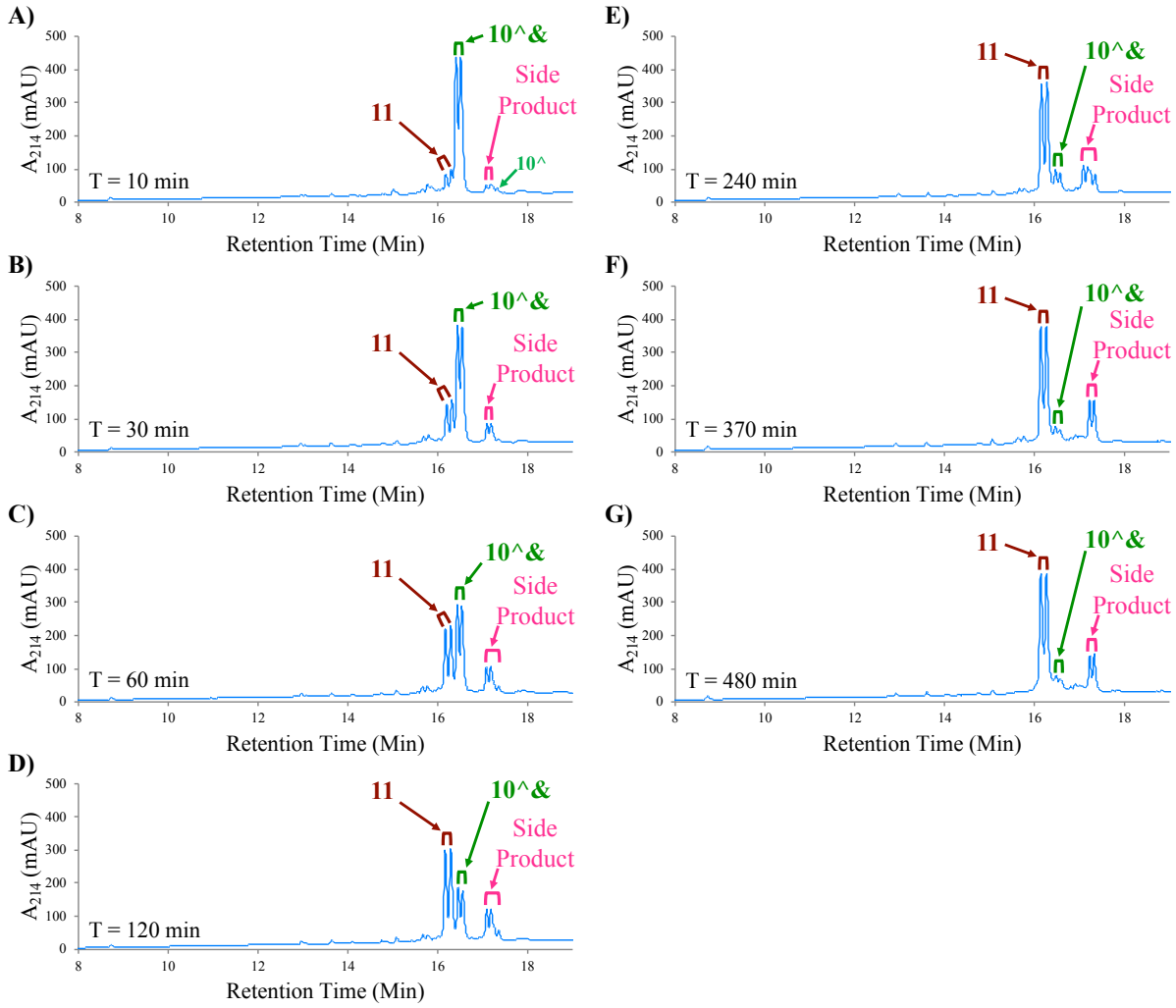


Figure S41: Time-course for the click-assisted native chemical ligation of 0.05 mM STEVEKENT-Far(CAN) Post-Click peptide (**10** not isolated after the SPAAC shown in Figures S35-36), performed under the same conditions as the 0.5 mM CAN reaction (100 mM MTG and 100 mM TCEP). **10^&** refers to S*t*Bu-deprotected **10** that contains a C-terminal MTG thioester. **10^** corresponds to S*t*Bu-protected **10** that contains a C-terminal MTG thioester. **11** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Although this reaction formed the desired **11**, a decent amount of an undesired side-product also formed. Note that the side-product originally has 2 peaks (10 min and 30 min), then forms three peaks (60 min, 120 min, and 240 min), and then goes back to 2 peaks that have an increased retention time compared to the original 2 peaks (370 min and 480 min). Initially, the side-product appears to result from the N-terminal Cys being capped in **10^&**, as mass spectrometry indicates this initial side-product has an observed mass of +74 Da compared to the expected mass of **10^&** (Figure S42(C)). This mass suggests that capping occurred via acylation from an MTG acyl donor. Over time, the capped **10^&** side-product reacts further, producing an observed mass of +146 Da compared to the expected **10^&** mass. This may be from the initial side-product undergoing a second acylation from an MTG acyl donor. Analytical method C was used for analyses.

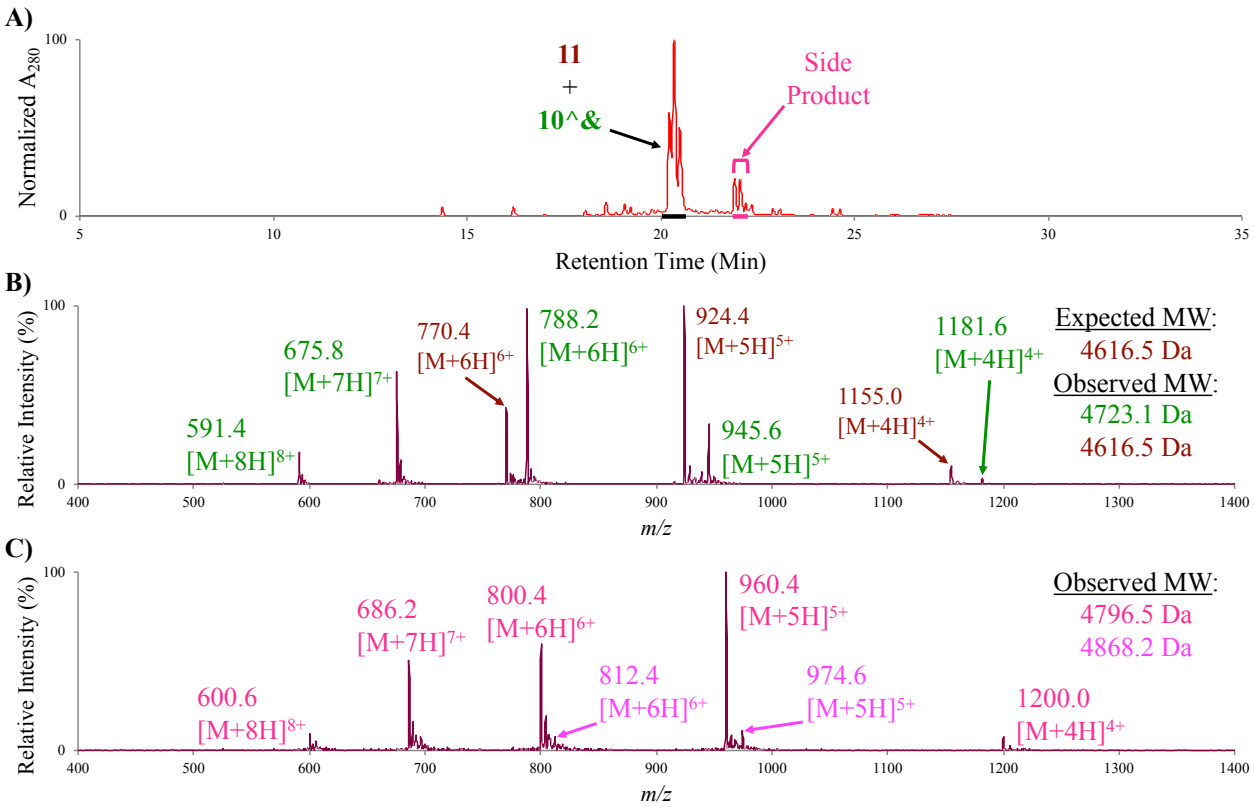


Figure S42: LC/MS analysis of the 60 min time point from the click-assisted native chemical ligation of STEVEKENT-Far(CAN) Post-Click peptide (**10**) shown in Figure S41 (0.05 mM **10**, 100 mM MTG, and 100 mM TCEP). (A) LC/MS chromatogram. **10^&** refers to S*t*Bu-deprotected **10** that contains a C-terminal MTG thioester. **11** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. (B) MS of the **11**/**10^&** peptide peaks (black bar in part A). The expected mass refers to **11**. A majority of the MS corresponds to **10^&** (observed mass of 4723.1 Da), and ions corresponding to **11** are also observed. (C) MS of the side-product peaks (pink bar in part A). The majority of the side-product corresponds to an observed mass that is +74 Da compared to the expected mass of **10^&** (4796.5 Da), which is likely **10^&** that underwent acylation from an MTG acyl donor. An observed mass that is +146 Da compared to the expected mass of **10^&** is also present (4868.2 Da). LC/MS analysis of the 480 min time point (not shown) revealed that the side-product almost completely converts to the +146 Da mass, indicating that the initial side-product (+74 Da) continues to react over time, and the increase in mass suggests that a second acylation may occur via an MTG acyl donor. LC/MS method F was used for analysis.

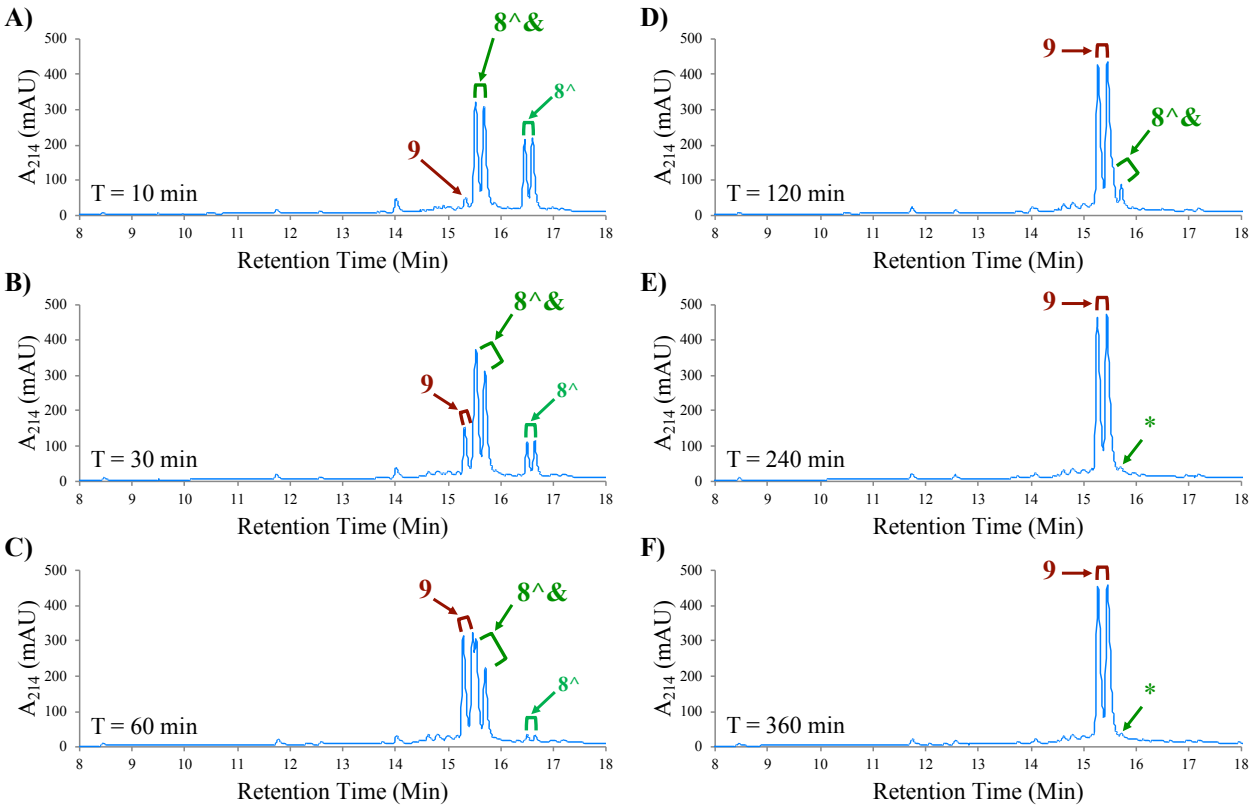


Figure S43: Time-course for one of the click-assisted native chemical ligations of 0.05 mM STEVEKENT-Close(CAN) Post-Click peptide (**8** not isolated after the SPAAC shown in Figure S30), performed with lower MTG and TCEP concentrations (10 mM MTG and 10 mM TCEP). **8^&** refers to S*t*Bu-deprotected **8** that contains a C-terminal MTG thioester. **8^** corresponds to S*t*Bu-protected **8** that contains a C-terminal MTG thioester. **9** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that **8^** was detected in (A), (B), and (C), unlike the 0.5 mM CAN reactions that only had **8^** in the first time point. Since this reaction has less TCEP than the 0.5 mM CAN reaction, S*t*Bu is not removed as quickly. The * in (E) and (F) are to note that the peak shown is not **8^&**, but rather a side-product that elutes near **8^&** (based on this peak not changing in (F) and no unligated material being detected by mass spectrometry). Note that the time points from the second CAN reaction of 0.05 mM **8** used for determining the kinetic rate are not shown. Since a regioisomer from both **8^&** and **9** nearly co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **8^&** and the left peak for **9**). In addition, only the right peak area of **8^** was used to calculate the total area of unligated peptide for the 10, 30, and 60 min time points. Analytical method C was used for analyses.

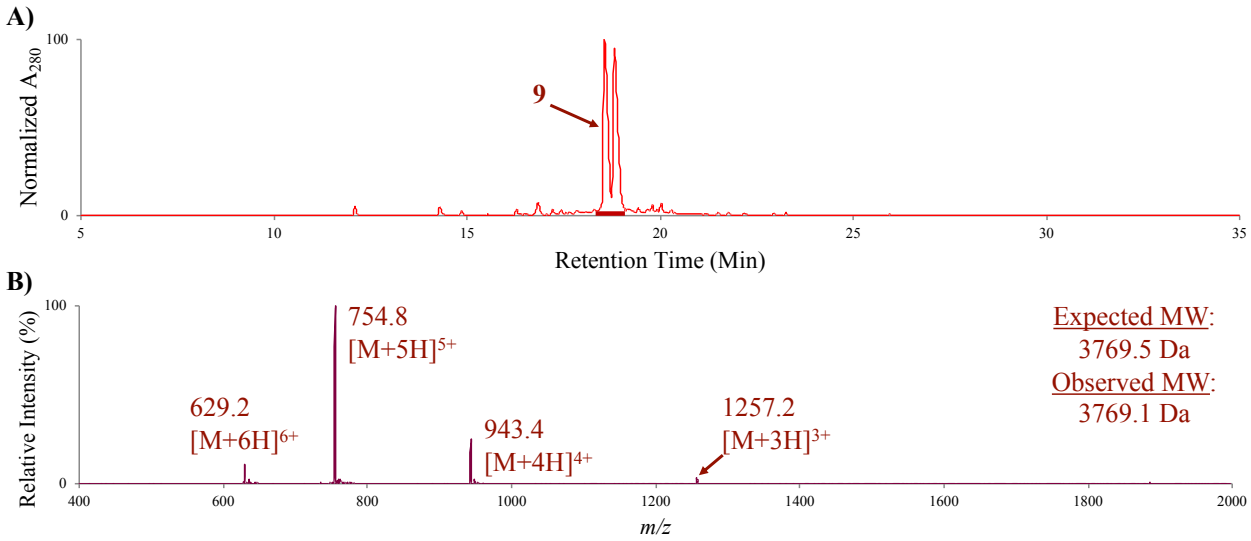


Figure S44: LC/MS analysis of the 360 min time point from the click-assisted native chemical ligation of STEVEKENT-Close(CAN) Post-Click peptide (**8**) shown in Figure S43 (0.05 mM **8**, 10 mM MTG, and 10 mM TCEP). (A) LC/MS chromatogram. The double peaks of the ligated product (**9**) are expected, as the triazole has two regioisomers. (B) MS of **9** (dark red bar in part A). LC/MS method F was used for analysis.

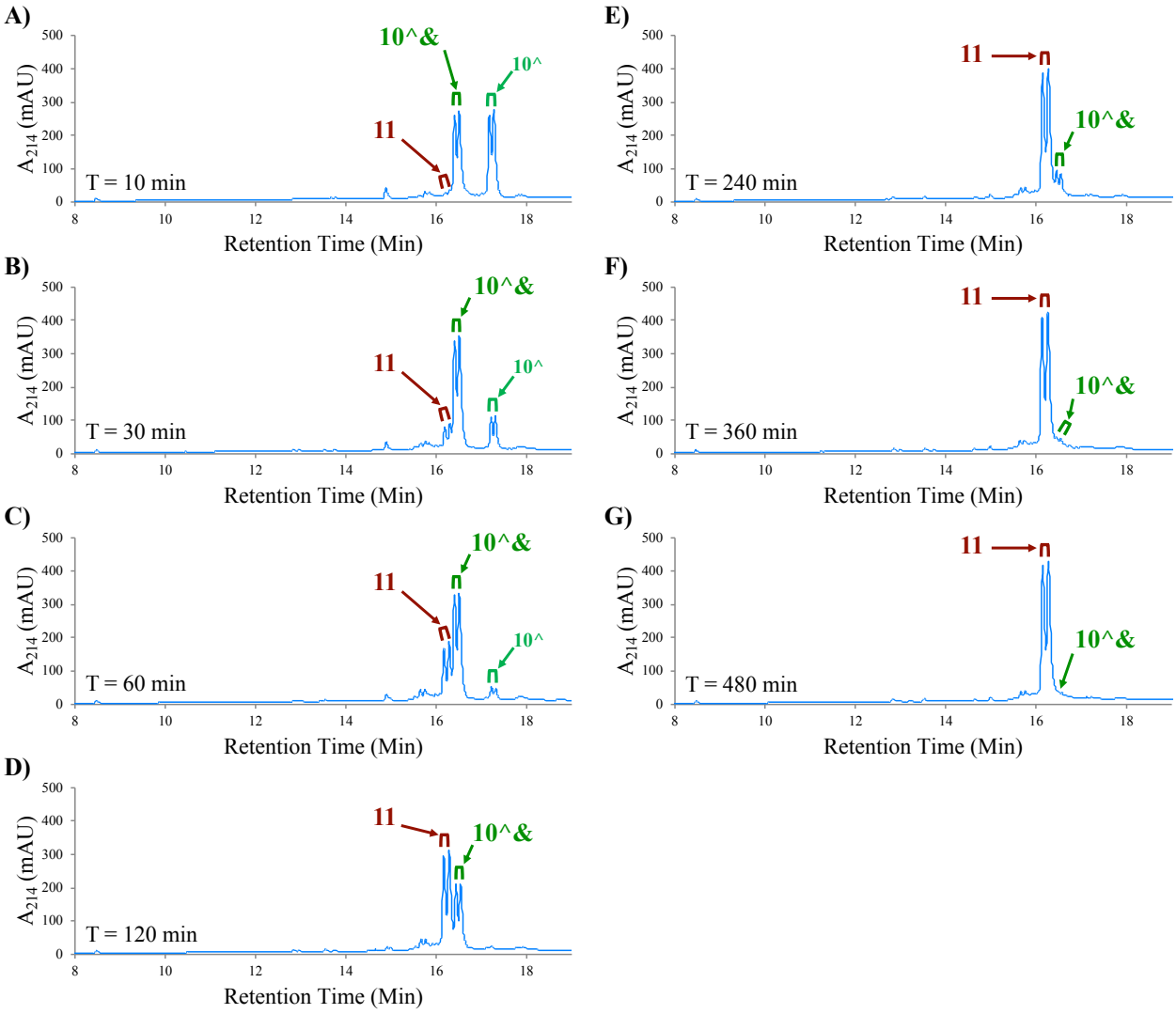


Figure S45: Time-course for one of the click-assisted native chemical ligations of 0.05 mM STEVEKENT-Far(CAN) Post-Click peptide (**10** not isolated after the SPAAC shown in Figures S35-36), performed with lower MTG and TCEP concentrations (10 mM MTG and 10 mM TCEP). **10^&** refers to S*t*Bu-deprotected **10** that contains a C-terminal MTG thioester. **10^** corresponds to S*t*Bu-protected **10** that contains a C-terminal MTG thioester. **11** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that **10^** was detected in (A), (B), and (C), unlike the 0.5 mM CAN reactions that only had **10^** in the first time point. Since this reaction has less TCEP than the 0.5 mM CAN reaction, S*t*Bu is not removed as quickly. The reaction was finished after 480 min, as nearly all of **10^&** reacted. Note that the time points from the second CAN reaction of 0.05 mM **10** used for determining the kinetic rate are not shown. Since a regioisomer from both **10^&** and **11** nearly co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **10^&** and the left peak for **11**). In addition, only the right peak area of **10^** was used to calculate the total area of unligated peptide for the 10, 30, and 60 min time points. Analytical method C was used for analyses.

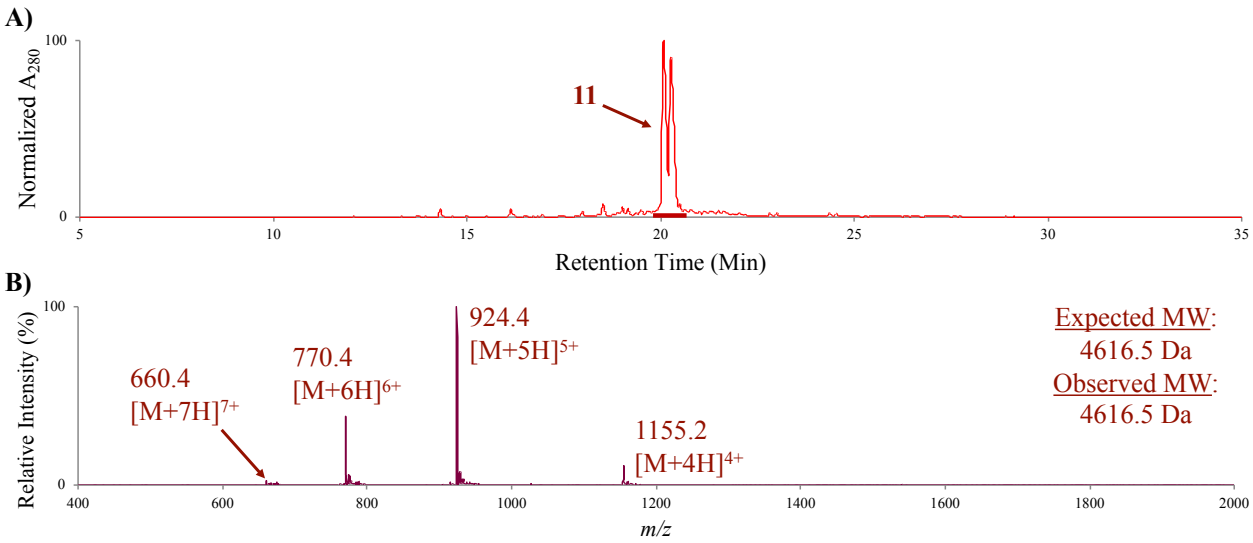


Figure S46: LC/MS analysis of the 480 min time point from the click-assisted native chemical ligation of STEVEKENT-Far(CAN) Post-Click peptide (**10**) shown in Figure S45 (0.05 mM **10**, 10 mM MTG, and 10 mM TCEP). (A) LC/MS chromatogram. The double peaks of the ligated product (**11**) are expected, as the triazole has two regioisomers. (B) MS of **11** (dark red bar in part A). LC/MS method F was used for analysis.

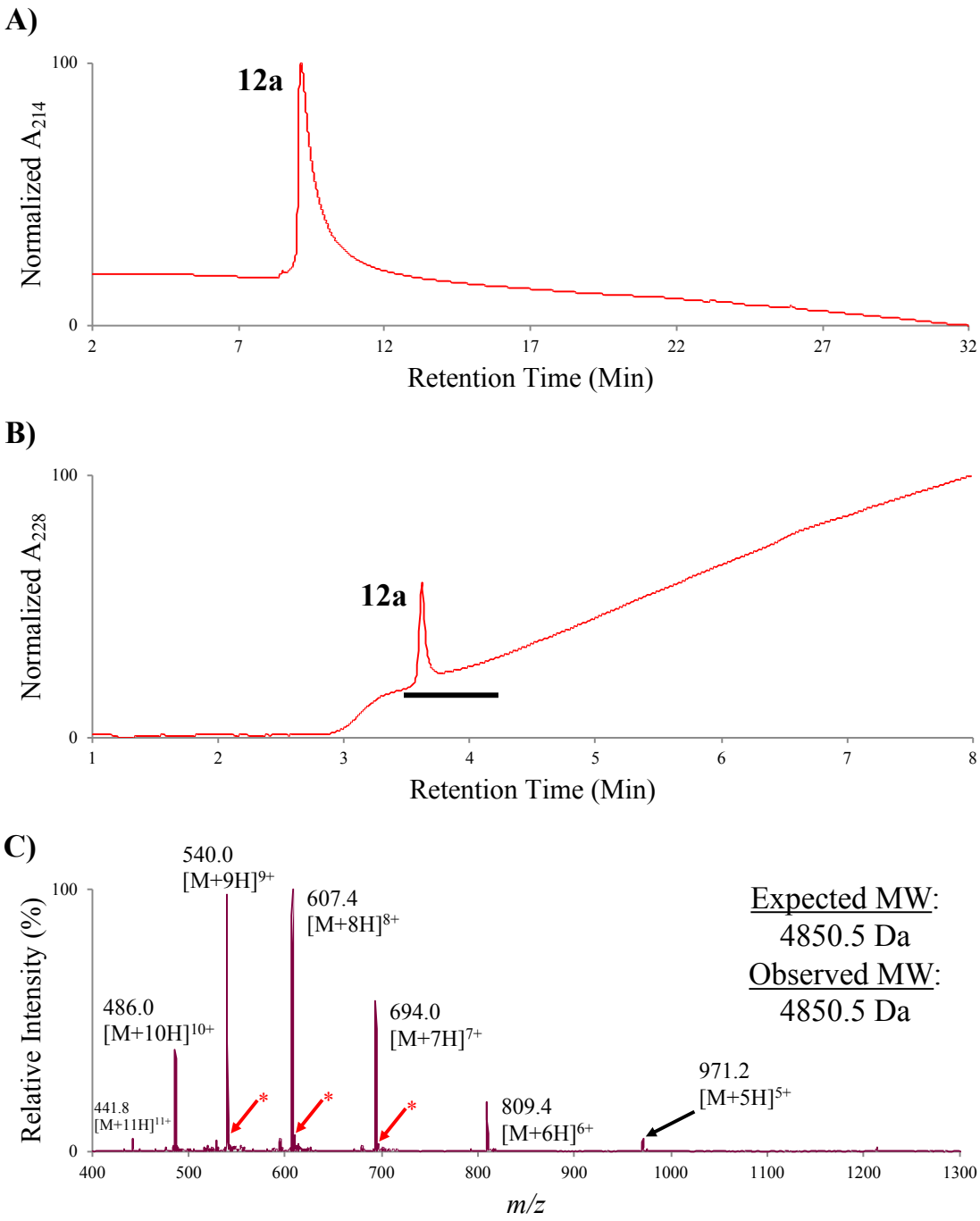


Figure S47: Pure L32-N(Ctrl) peptide (**12a**). (A) HPLC chromatogram. (B) LC/MS chromatogram. As Tyr and Trp are not in **12a**, A_{228} was used (note that the A_{228} increases over time due to acetonitrile being added). (C) MS of pure **12a** (black bar in part B). * corresponds to an observed mass of 4866.3 Da, which is likely oxidized **12a**. This peptide was synthesized at 30 µmol scale, cleaved with cocktail containing EDT and NH_4I (92.5% TFA, 2.5% TIS, 2.5% H_2O, 2.5% EDT, and ~100 mg NH_4I for 3 h), and purified using purification methods K-P. Analytical method D and LC/MS method C were used for analyses. The isolated yield of pure **12a** was 11%.

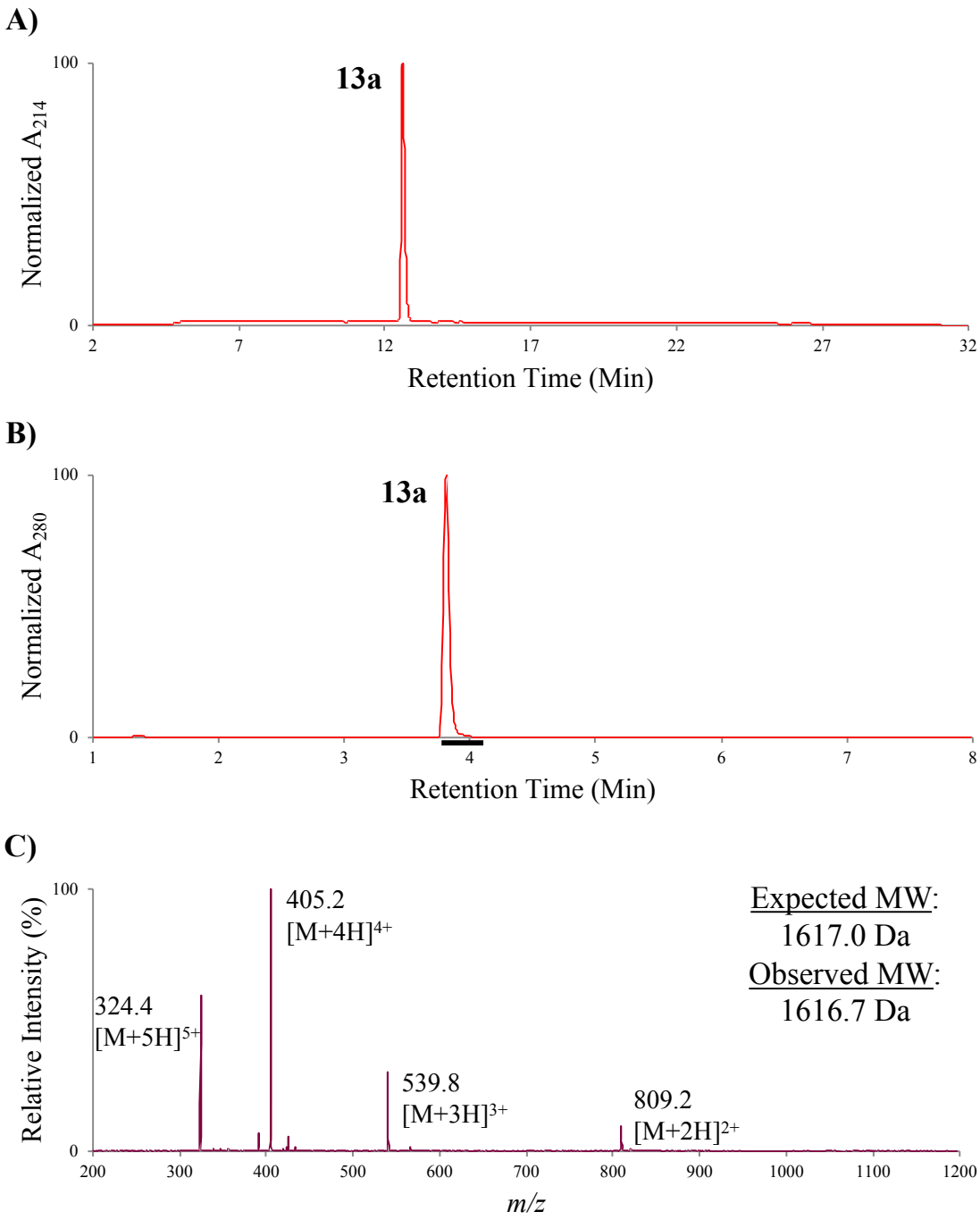


Figure S48: Pure L32-C(Ctrl) peptide (**13a**). (A) HPLC chromatogram. (B) LC/MS chromatogram. (C) MS of pure **13a** (black bar in part B). Note that the fragmentation voltage was lowered for this LC/MS run (50 V), and the mass scan range was changed to 200 – 1,200 *m/z*. These parameters were used due to fragmentation of the *tert*-butyl group in S*t*Bu being observed when the standard LC/MS parameters (90 V and 400 – 2,000 *m/z*) were used on **13a**. This peptide was synthesized at 30 μmol scale, cleaved under standard conditions, and purified using purification methods Q and R. Analytical method E and LC/MS method G were used for analyses. The isolated yield of pure **13a** was 44%.

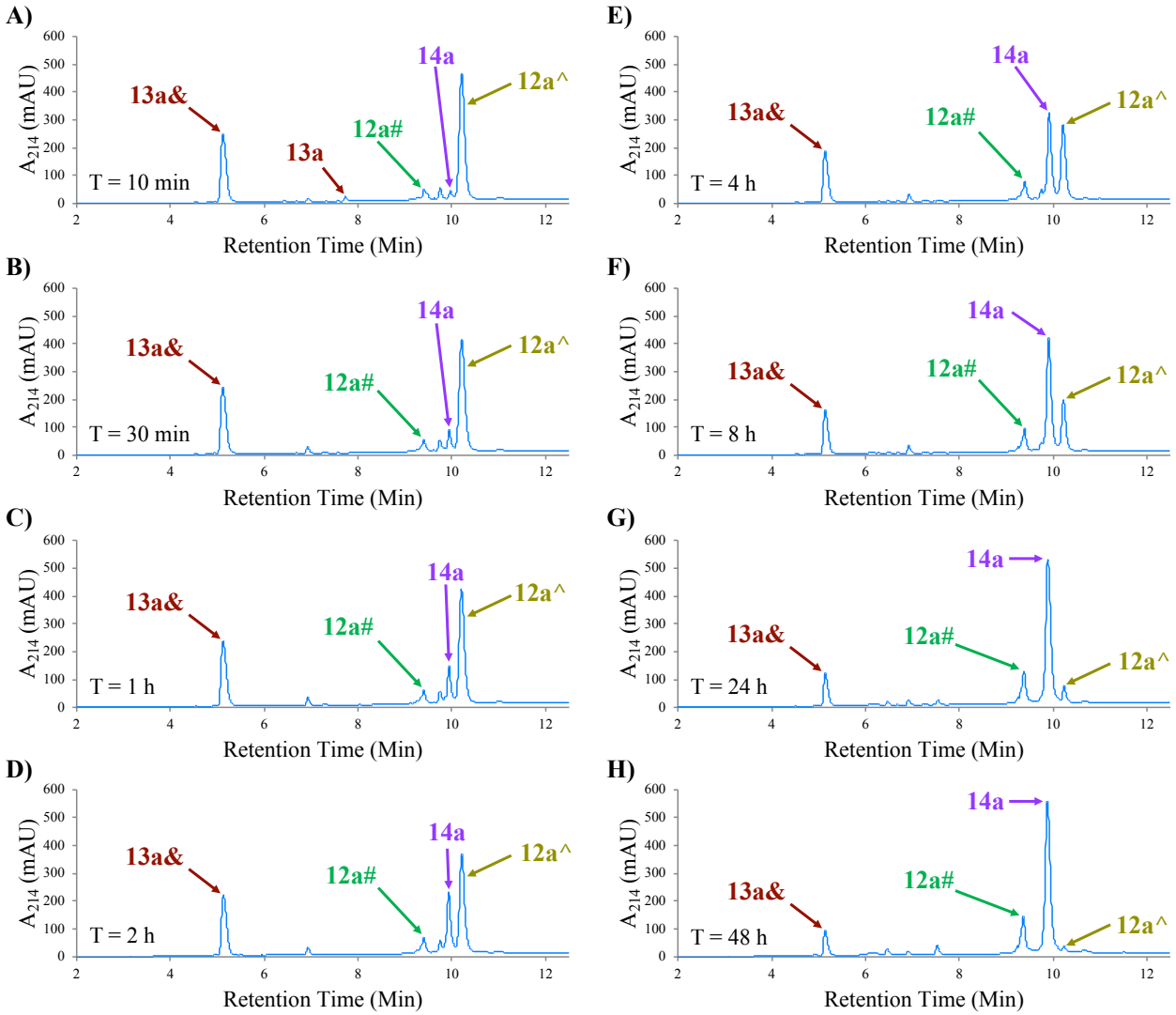


Figure S49: Time-course of the non-templated native chemical ligation between L32-N(Ctrl) (**12a**; 0.4 mM) and L32-C(Ctrl) (**13a**; 0.5 mM) peptides (same reaction as time point shown in Figure 7). **13a&** refers to S*t*Bu-deprotected **13a**. **12a^** refers to **12a** containing a C-terminal MTG thioester. **12a#** corresponds to **12a^** that underwent thioester hydrolysis during the NCL. **14a** corresponds to the ligated product (L32-Met). Note that this is one of two NCL's performed for kinetic calculations and that the traces for the other reaction are not shown. Time points refer to when MTG was added to the reaction. The reaction was finished after 48 h, as nearly all of **12a^** reacted. Analytical method B was used for analyses. Scheme S3 shows the detailed reaction conditions for this NCL.

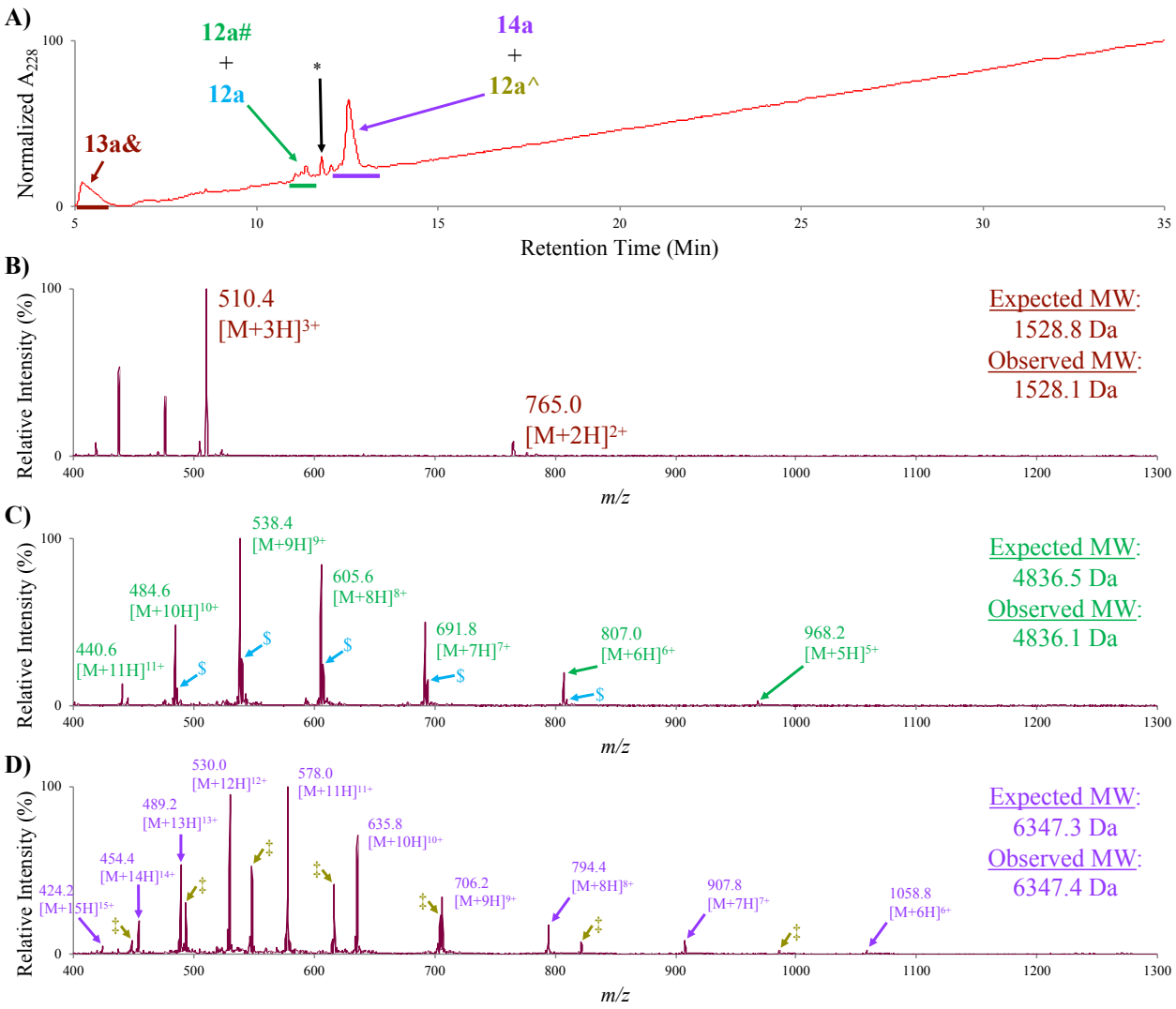


Figure S50: LC/MS analysis of the 8 h time point from the non-templated NCL between L32-N(Ctrl) (**12a**; 0.4 mM) and L32-C(Ctrl) (**13a**; 0.5 mM) shown in Figure S49. (A) LC/MS chromatogram (note that the A_{228} increases over time due to acetonitrile being added). **13a&** refers to S*t*Bu-deprotected **13a**. **12a^** refers to **12a** containing a C-terminal MTG thioester. **12a#** corresponds to **12a^** that underwent thioester hydrolysis during the NCL. **14a** corresponds to the ligated product (L32-Met). * refers to a **13a&** side-product (+74 Da compared to the expected mass of **13a&**). (B) MS of the **13a&** peak (dark red bar in part A). (C) MS of the **12a#**/**12a** peaks (green bar in part A). The expected mass refers to **12a#**. $ refers to the observed mass of **12a** (4851.4 Da). (D) MS of the **14a**/**12a^** peaks (purple bar in part A). The expected mass refers to **14a**. ‡ refers to the observed mass of **12a^** (4924.5 Da). LC/MS method E was used for analysis.

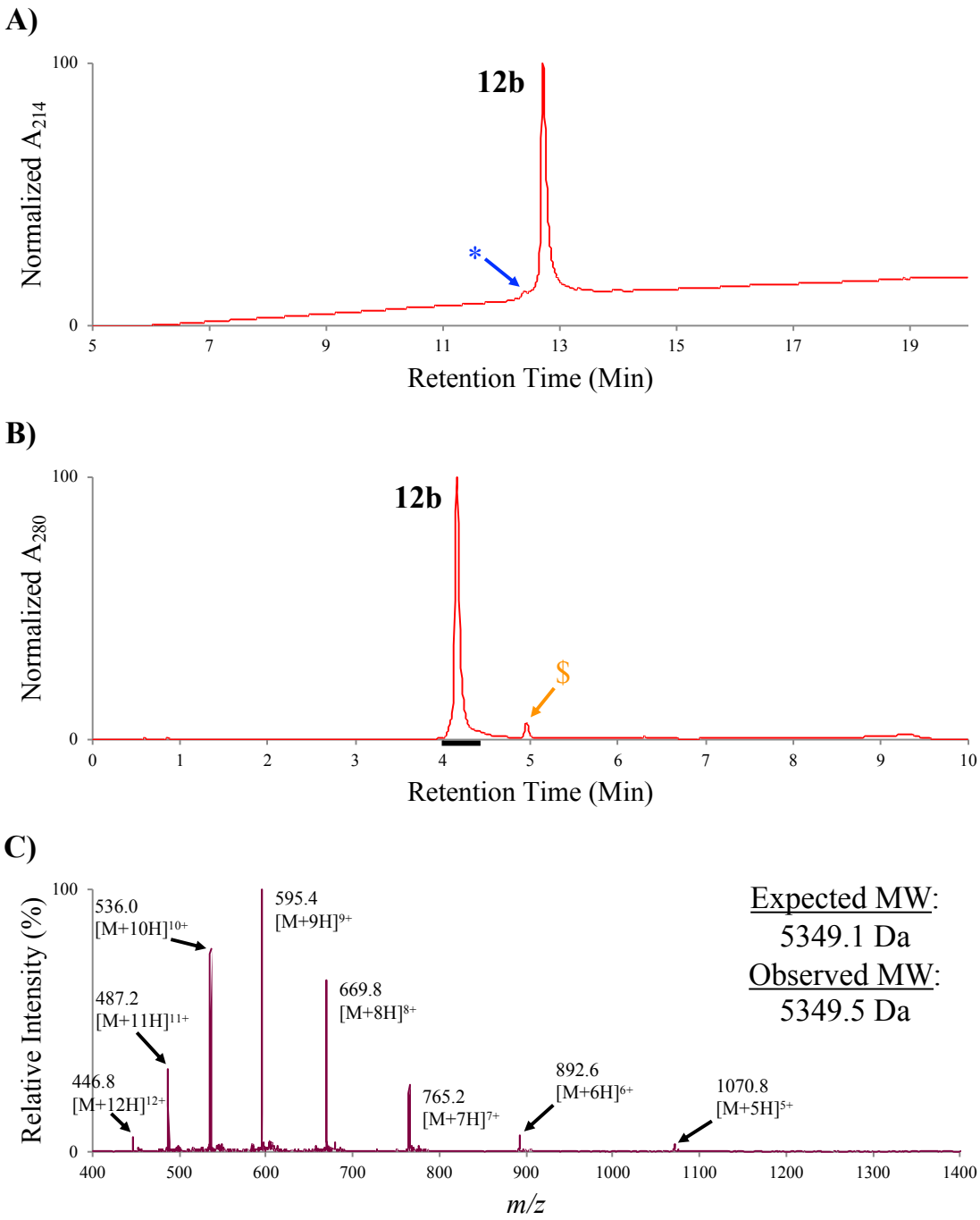


Figure S51: Pure L32-N-Close(CAN) peptide (**12b**). (A) HPLC chromatogram. * corresponds to an unrelated peptide that was carried over from a previous run on the HPLC column. (B) LC/MS chromatogram. $ corresponds to a non-peptide contaminant (no absorbance detected at 214 nm and no MS observed within this peak). (C) MS of pure **12b** (black bar in part B). This peptide was synthesized at 30 µmol scale, cleaved under standard conditions, and purified using purification methods S-U. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **12b** was 4.5%.

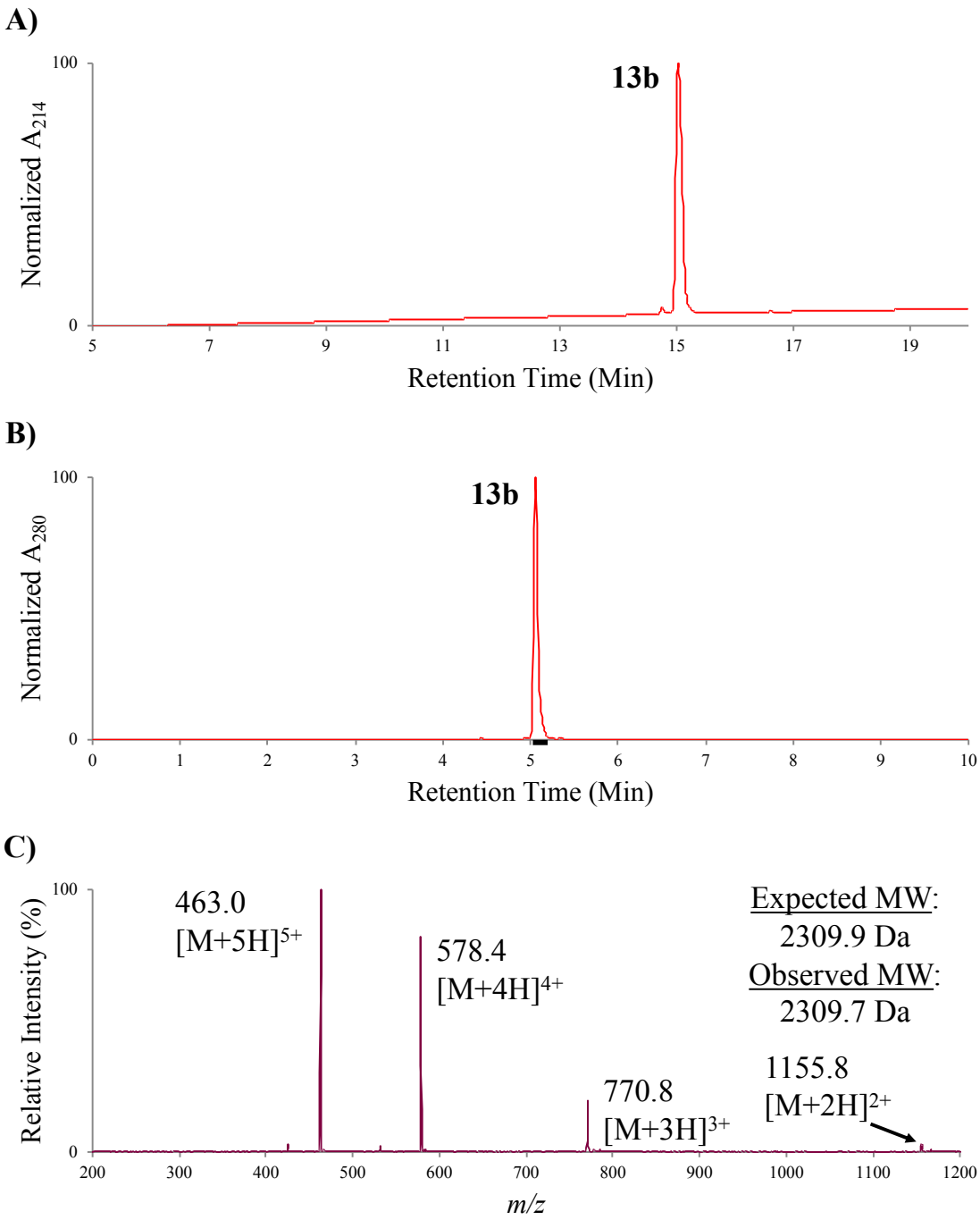


Figure S52: Pure L32-C(CAN) peptide (**13b**). (A) HPLC chromatogram. (B) LC/MS chromatogram. (C) MS of pure **13b** (black bar in part B). Note that the fragmentation voltage was lowered for this LC/MS run (50 V), and the mass scan range was changed to 200 – 1,200 *m/z*. These parameters were used in order to avoid fragmentation of the S*t*Bu group, as observed in the L32-C(Ctrl) peptide (**13a**; Figure S48). This peptide was synthesized at 30 µmol scale, cleaved under standard conditions with 5 equiv of $(MeCN)_4CuBF_4$, and purified using purification methods V-X. Analytical method C and LC/MS method G were used for analyses. The isolated yield of pure **13b** was 29%.

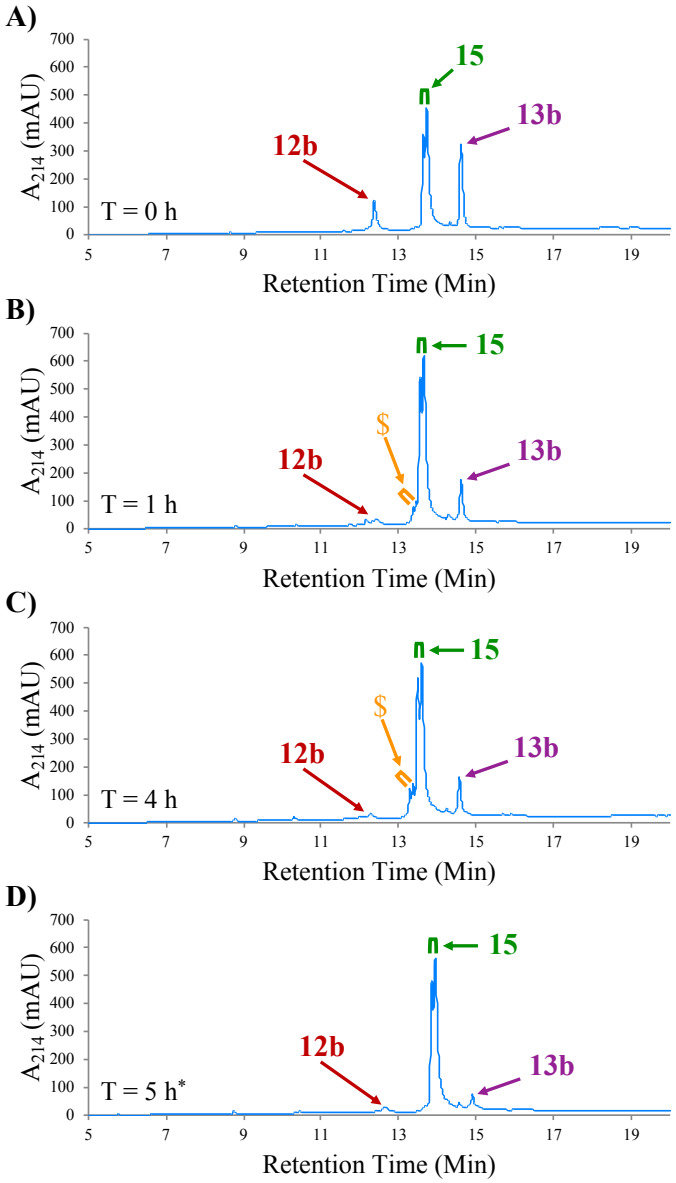


Figure S53: Time-course for the SPAAC between L32-N-Close(CAN) (**12b**; 1 mM) and L32-C(CAN) (**13b**; 1 mM). The double peaks of the triazole peptide (**15**) are expected, as the triazole has two regioisomers. $ in (B) and (C) refer to carry over of the triazole product from the SPAAC between L32-N-Far(CAN) (**12c**) and **13b**, as this reaction was being analyzed on the same HPLC column on the same day (Figure S61). After 4 h of SPAAC at 37°C (parts A-C), the reaction was placed in -80°C, but a significant amount of unreacted **13b** remained. A few weeks later, the reaction was taken out of -80°C, warmed to rt, and more **12b** was added. The reaction was placed at 37°C for 1 h, and then the reaction was placed back into -80°C until the CAN reactions were performed, as most of the **13b** peptide reacted during the additional 1 h (part D). The * in (D) is to note the addition of more **12b** to the reaction. The retention times are shifted in (D) due to slight differences in HPLC buffer composition and the HPLC experiencing high backpressure from a malfunctioning part in parts A-C. Note that (A) and (D) are also shown in Figure 9. Analytical method C was used for analyses.

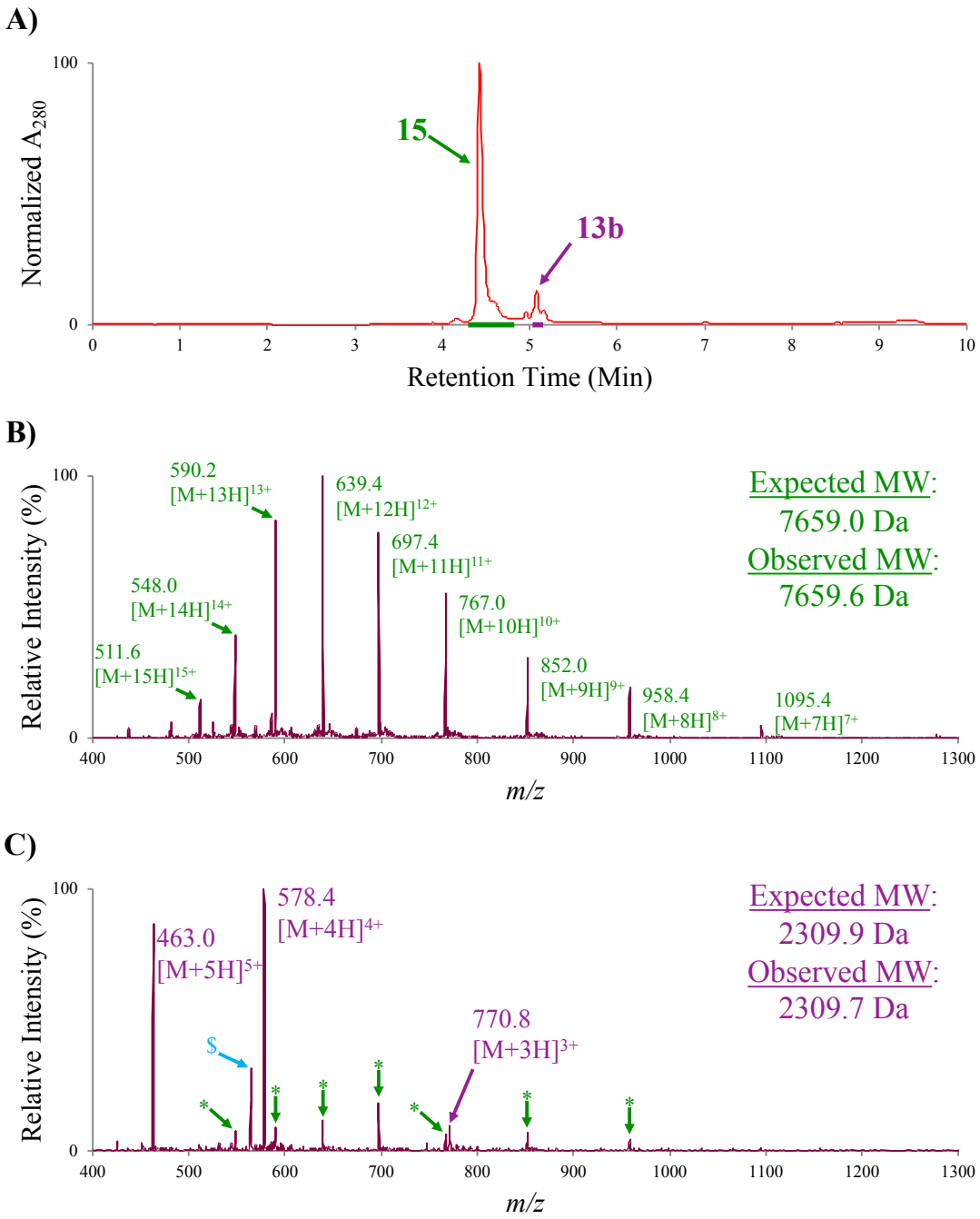


Figure S54: LC/MS analysis of the 5 h time point from the SPAAC between L32-N-Close(CAN) (**12b**) and L32-C(CAN) (**13b**) shown in Figure S53. (A) LC/MS chromatogram. (B) MS of the triazole-linked peptide (**15**; green bar in part A). (C) MS of the unreacted **13b** peptide (purple bar in part A). $ corresponds to an observed mass of 2253.6 Da, and this is likely from **13b** being fragmented at the *tert*-butyl group of S*t*Bu. The fragmentation voltage typically used for our analyses (90 V) was likely too high for this peptide, based on results observed for L32-C(Ctrl) (**13a**; discussed in Figure S48). * corresponds to **15** (observed mass of 7659.3 Da). C-terminal hydrazide peptides can slowly tail off of HPLC columns, which is likely why ions for **15** were observed in the **13b** peak. LC/MS method C was used for analysis.

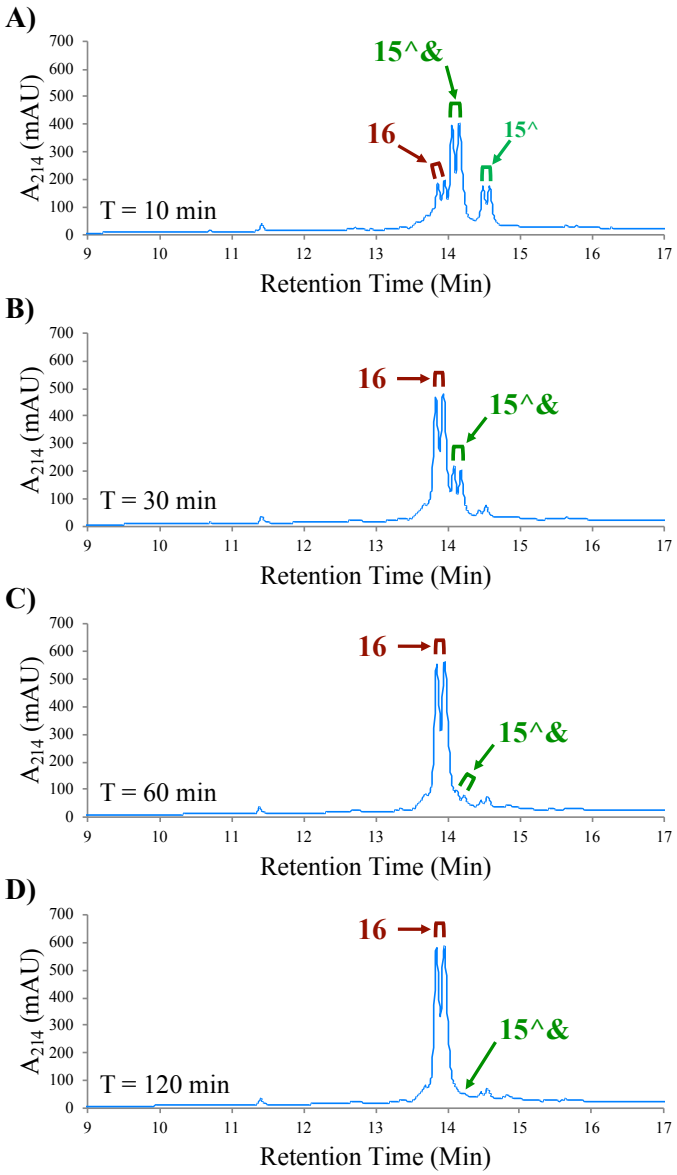


Figure S55: Time-course for one of the click-assisted native chemical ligations of 0.3 mM L32-Close-Post-Click peptide (**15** not isolated after the SPAAC shown in Figures S53-54). **15^&** refers to S*t*Bu-deprotected **15** that contains a C-terminal MTG thioester. **15^** corresponds to S*t*Bu-protected **15** that contains a C-terminal MTG thioester. **16** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Note that (D) is also shown in Figure 9. Since nearly all of **15^&** was reacted in (D), the CAN reaction was finished after 2 h. Shortly after the 2 h time point was taken, the reaction was placed in -20°C until the desulfurization reaction was performed. Note that a second CAN of 0.3 mM **15** was performed for calculating kinetics, but the time points for this second CAN are not shown. Since a regioisomer from both **15^&** and **16** nearly co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **15^&** and the left peak for **16**). In addition, only the right peak area of **15^** was used to calculate the total area of unligated peptide for the 10 min time point. Analytical method C was used for analyses.

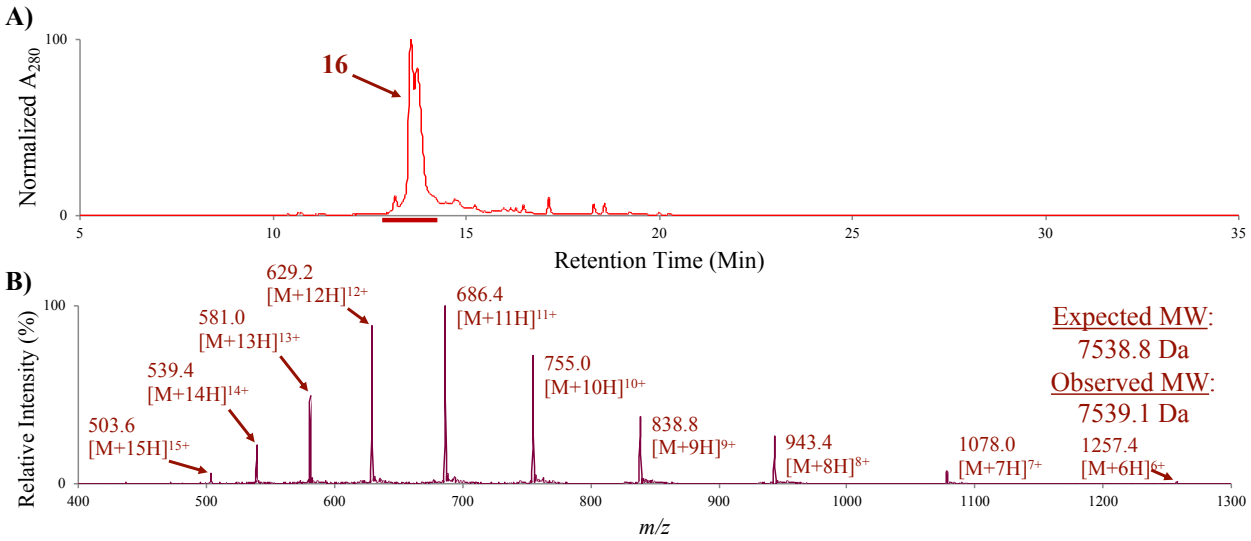


Figure S56: LC/MS analysis of the 120 min time point from the click-assisted native chemical ligation of 0.3 mM L32-Close-Post-Click peptide (**15**) shown in Figure S55. (A) LC/MS chromatogram. The double peaks of the ligated product (**16**) are expected, as the triazole has two regioisomers. (B) MS of **16** (dark red bar in part A). LC/MS method F was used for analysis.

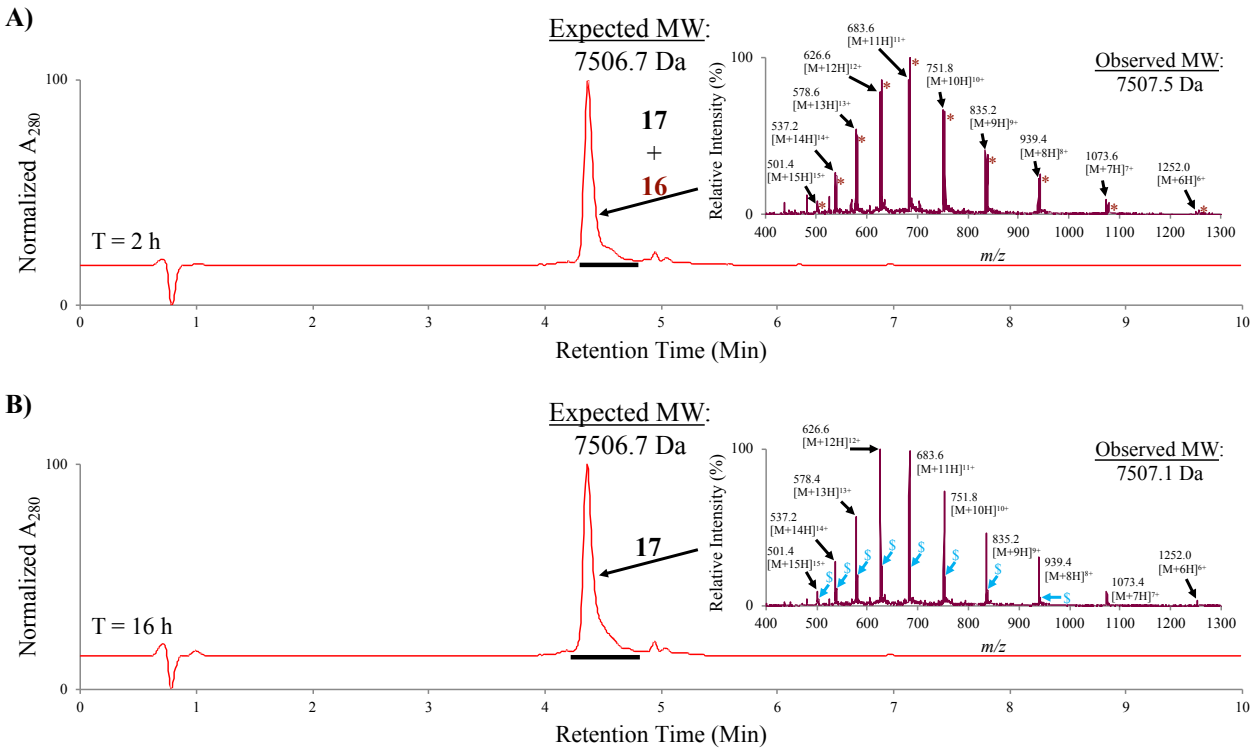


Figure S57: Time-course for the desulfurization of the L32-Close-Post-NCL peptide (**16** not isolated after the CAN shown in Figures S55-56). (A) LC/MS chromatogram of the 2 h desulfurization time point. MS of the main peak (black bar) is shown. Although the desulfurized peptide (**17**) is observed, there is a significant amount of Cys-containing **16** still remaining (ions with *; observed mass of 7539.5 Da). (B) LC/MS chromatogram of the 16 h desulfurization time point. MS of the main peak (black bar) is shown. $ corresponds to an observed mass of 7524.3 Da, which is likely hydrolysis of a Ddap group (+18 Da compared to the expected mass of **17**). The desulfurization was finished after 16 h, as no Cys-containing **16** is observed in the MS. Shortly after the 16 h time point was taken, the desulfurization reaction was placed in -80°C until Ddap linker cleavage was performed. Note that the HPLC chromatogram of the 16 h time point is shown in Figure 9. LC/MS method C was used for analyses.

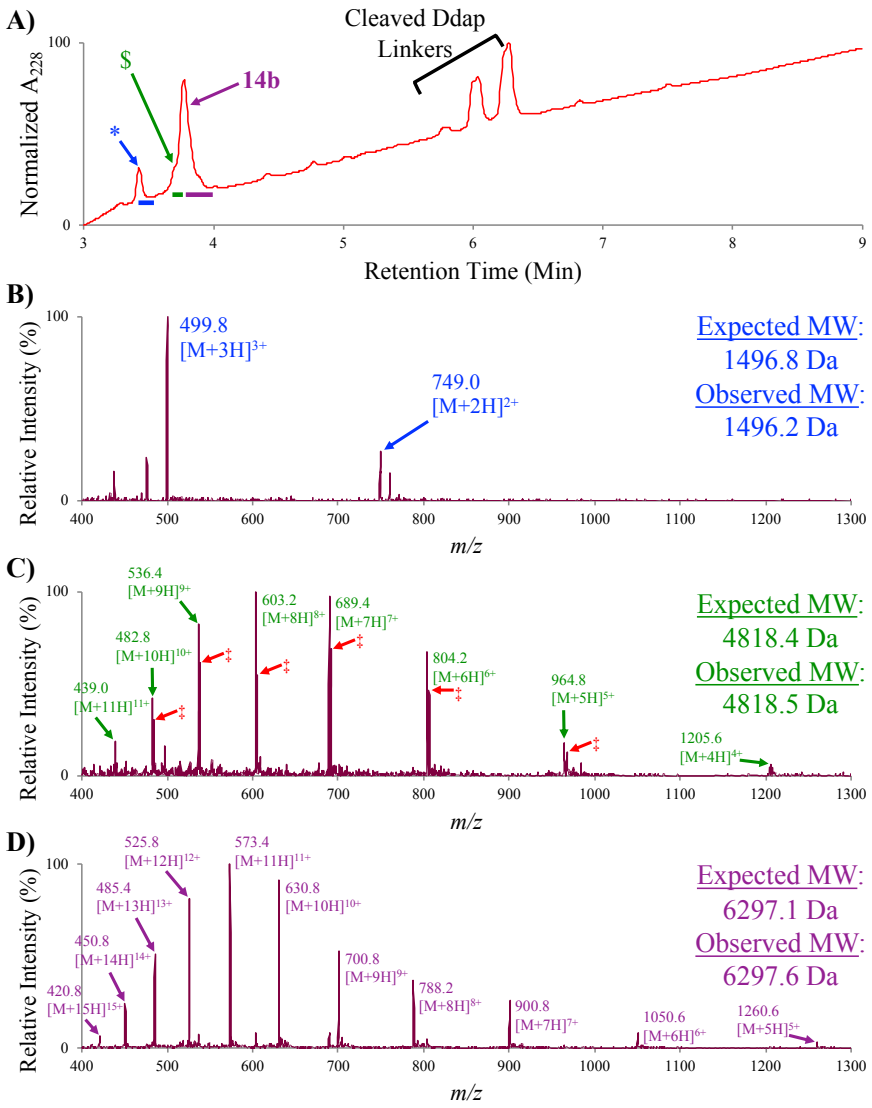


Figure S58: LC/MS analysis of the 1 h time point from the Ddap cleavage reaction of L32-Close-Post-Desulfurization peptide (**17** not isolated after the desulfurization shown in Figure S57). (A) LC/MS chromatogram (note that the A_{228} increases over time due to acetonitrile being added). The chromatogram starts at 3 min in order to omit the buffer salt peaks. * refers to desulfurized L32-C(CAN) peptide (**13b**) without the Ddap linker. $ corresponds to L32-N-Close(CAN) peptide (**12b**) without the Ddap linker, containing either a C-terminal hydroxyl or hydrazide. **14b** refers to full-length L32-Nle. Since Ddap hydrolysis was observed during desulfurization (Figure S57), there are more peaks associated with cleaved Ddap linkers compared to the STEVEKENT Ddap linker cleavage (Figure S28). (B) MS of the desulfurized L32-C(CAN) peptide (**13b**) without the Ddap linker (blue bar in part A). (C) MS of the L32-N-Close(CAN) peptide (**12b**) without the Ddap linker (green bar in part A). The expected mass refers to peptide that underwent thioester hydrolysis during CAN, and the main mass observed corresponds to this peptide. ‡ corresponds to an observed mass of 4832.9 Da, which is C-terminal hydrazide peptide that failed to activate during CAN. (D) MS of **14b** (purple bar in part A). Note that the HPLC chromatogram of the Ddap cleavage end point (1.5 h) is shown in Figure 9. LC/MS method C was used for analysis.

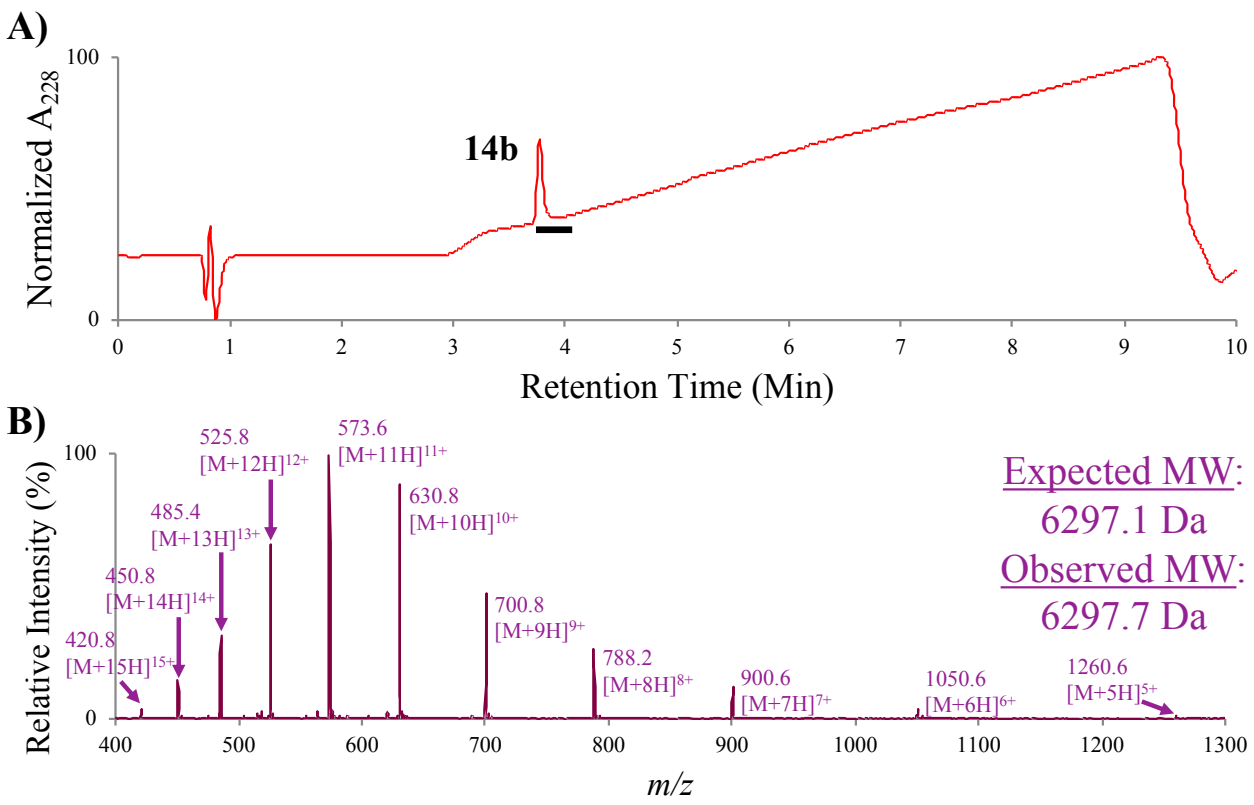


Figure S59: LC/MS analysis of the purified L32-Nle peptide (**14b**) following L32-Close SPAAC, CAN, desulfurization, and Ddap linker cleavage (Figures S53-58; Scheme S4 shows specific details for all four reactions). (A) LC/MS chromatogram (note that the A_{228} increases over time due to acetonitrile being added). (B) MS of pure **14b** (black bar in part A). This is the same MS used to generate the deconvoluted MS shown in Figure 9. The observed masses are slightly different, as manual mass calculation based on the observed charge states was used for this supplemental figure, whereas the UniDec software was used to calculate the deconvoluted MS shown in Figure 9.[7] Note that Figure 9 also shows the HPLC chromatogram of pure **14b**. This peptide was purified using purification method Y. LC/MS method C was used for analysis.

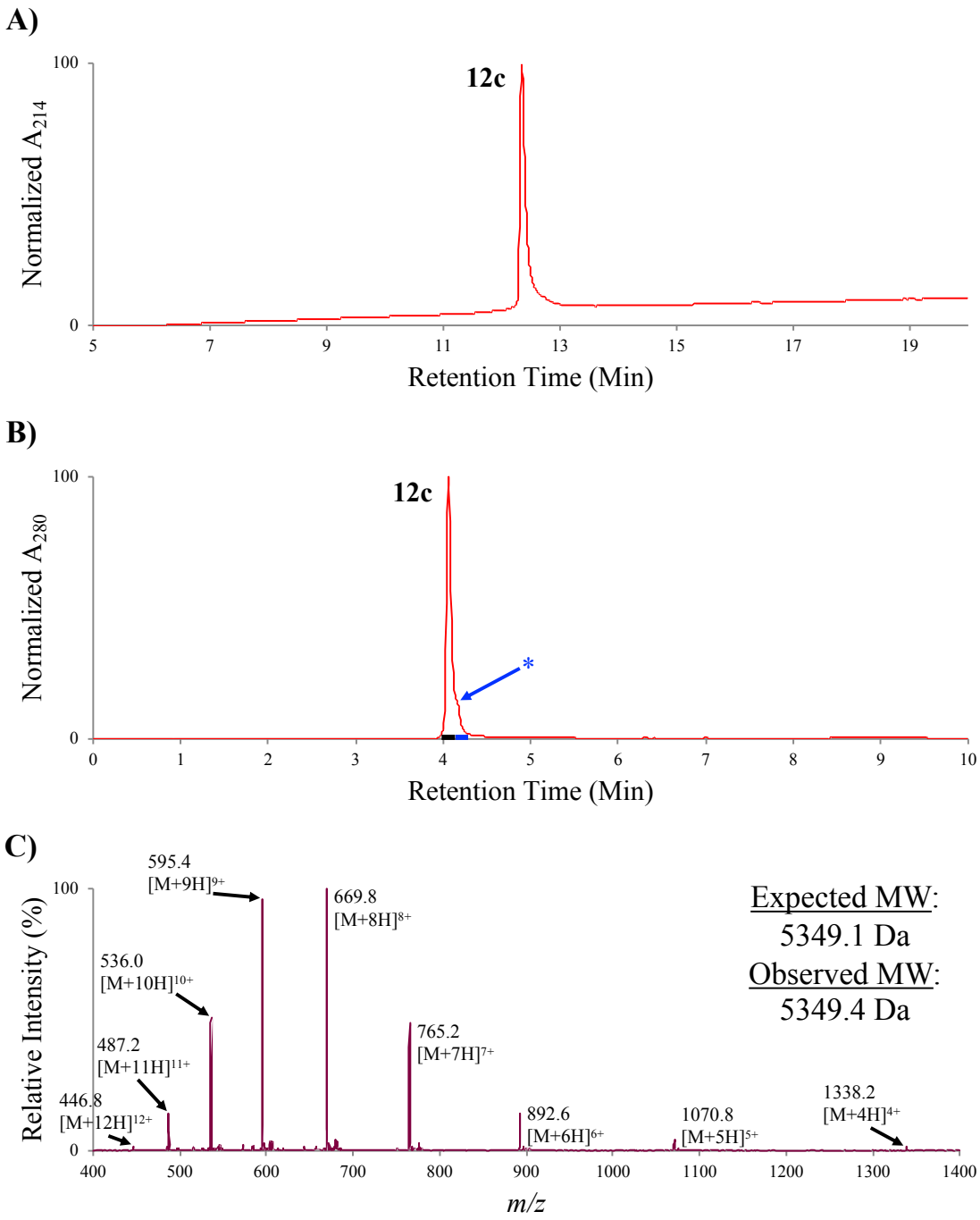


Figure S60: Pure L32-N-Far(CAN) peptide (**12c**). (A) HPLC chromatogram. (B) LC/MS chromatogram. * corresponds to carry over of the pure L32-N-Close(CAN) peptide (**12b**; observed mass of 5348.7 Da), as pure **12b** was run on the LC/MS column immediately before pure **12c** was run on the column. (C) MS of pure **12c** (black bar in part B). This peptide was synthesized at 30 μmol scale, cleaved under standard conditions, and purified using purification method Z. Analytical method C and LC/MS method C were used for analyses. The isolated yield of pure **12c** was 6.8%.

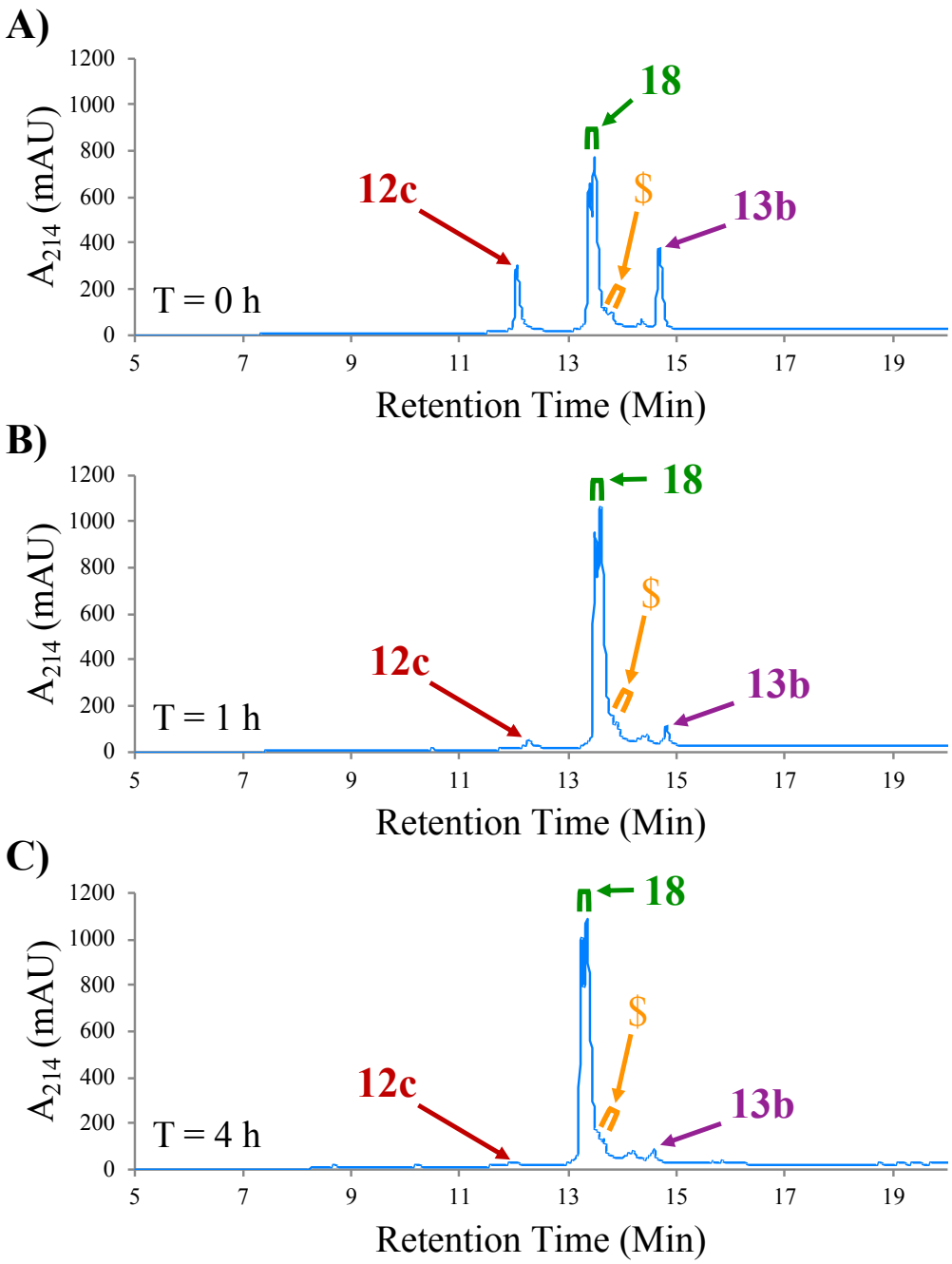


Figure S61: Time-course for the SPAAC between L32-N-Far(CAN) (**12c**; 1.6 mM) and L32-C(CAN) (**13b**; 1.7 mM). The double peaks of the triazole product (**18**) are expected, as triazole has two regioisomers. $ refers to carry over of the triazole product from the SPAAC between L32-N-Close(CAN) peptide (**12b**) and **13b**, as this reaction was being analyzed on the same HPLC column on the same day (Figure S53). SPAAC was finished after 4 h, as all of **12c** reacted and only a very small amount of unreacted **13b** remained. Shortly after the 4 h time point was taken, the reaction was placed in -80°C until the CAN reactions were performed. The retention times are slightly shifted for each time point due to high HPLC backpressure caused by a malfunctioning part. Analytical method C was used for analyses.

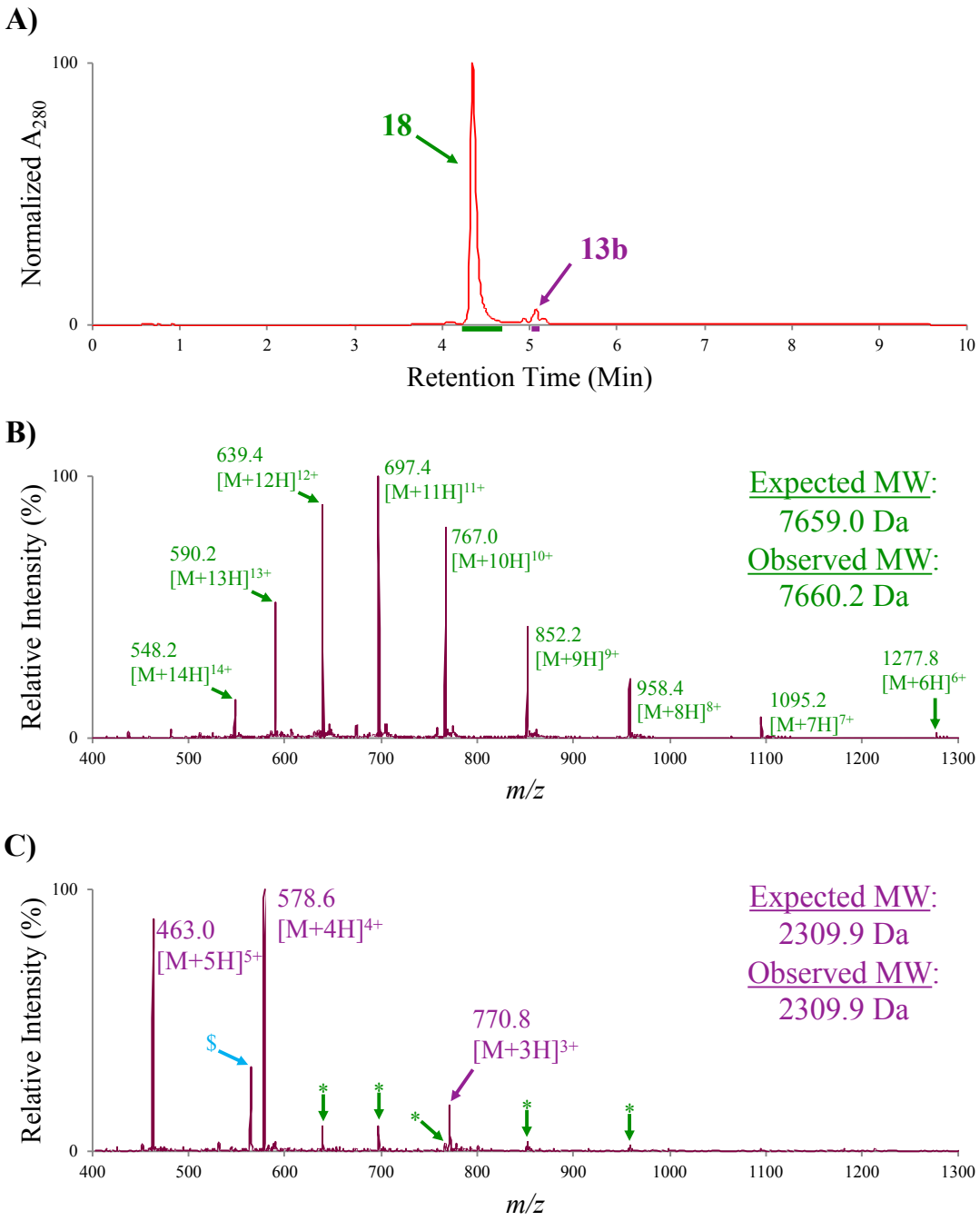


Figure S62: LC/MS analysis of the 4 h time point from the SPAAC between L32-N-Far(CAN) (**12c**) and L32-C(CAN) (**13b**) shown in Figure S61. (A) LC/MS chromatogram. (B) MS of the triazole-linked peptide (**18**; green bar in part A). (C) MS of the unreacted **13b** (purple bar in part A). $ corresponds to an observed mass of 2254.4 Da, and this is likely from **13b** being fragmented at the *tert*-butyl group of S*t*Bu. The fragmentation voltage typically used for our analyses (90 V) was likely too high for this peptide, based on results observed for L32-C(Ctrl) (**13a**; discussed in Figure S48). * corresponds to **18** (observed mass of 7659.8 Da). C-terminal hydrazide peptides can slowly tail off of HPLC columns, which is likely why ions for **18** were observed in the **13b** peak. LC/MS method C was used for analysis.

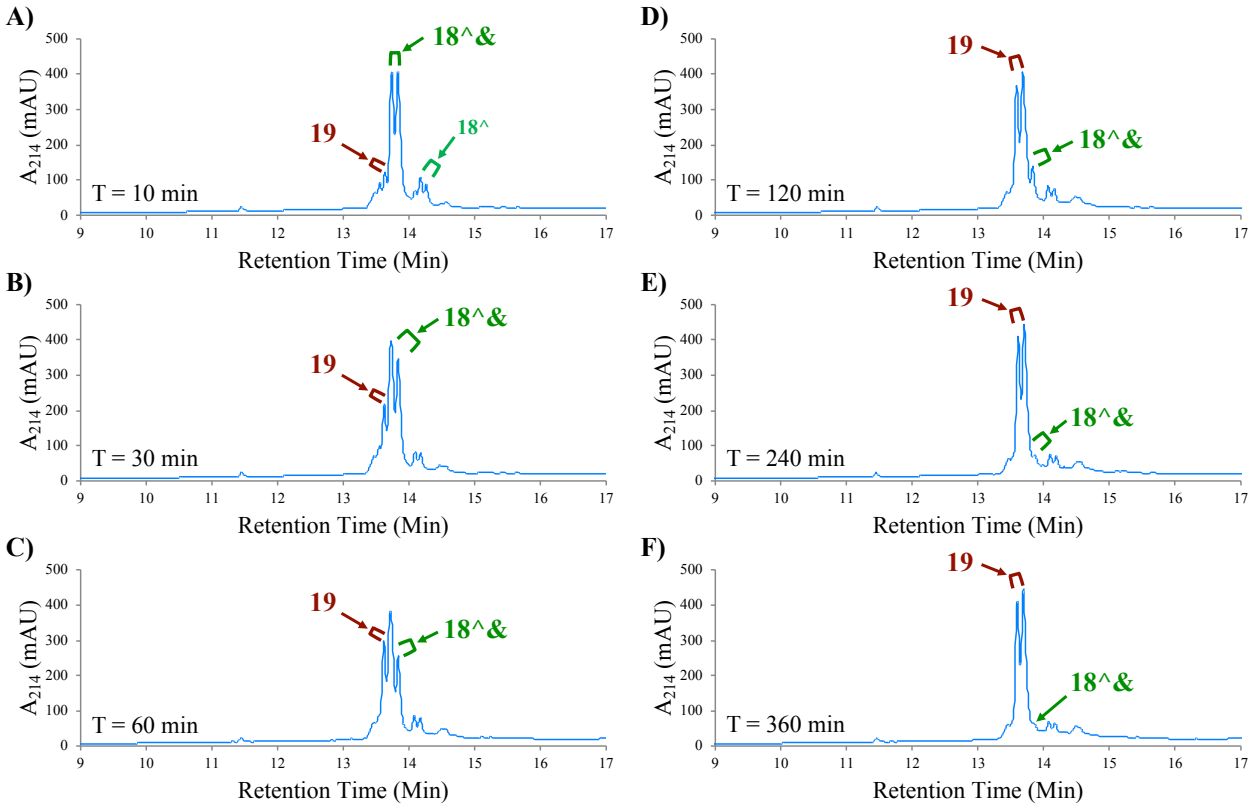


Figure S63: Time-course for one of the click-assisted native chemical ligations of 0.3 mM L32-Far-Post-Click peptide (**18** not isolated after the SPAAC shown in Figures S61-62). **18^&** refers to S*t*Bu-deprotected **18** that contains a C-terminal MTG thioester. **18^** corresponds to S*t*Bu-protected **18** that contains a C-terminal MTG thioester. **19** refers to the ligated product. The double peaks of the various peptides are expected, as the triazole has two regioisomers. Time points refer to when MTG was added to the reaction. Since nearly all of **18^&** was reacted in (F), the CAN reaction was finished after 6 h. Note that a second CAN of 0.3 mM **18** was performed for calculating kinetics, but the time points for this second CAN are not shown. Since a regioisomer from both **18^&** and **19** co-elute with each other, only the peak areas of regioisomers that do not co-elute were used for kinetic rate analyses (i.e., the right peak for **18^&** and the left peak for **19**). In addition, only the right peak area of **18^** was used to calculate the total area of unligated peptide for the 10 min time point. Analytical method C was used for analyses.

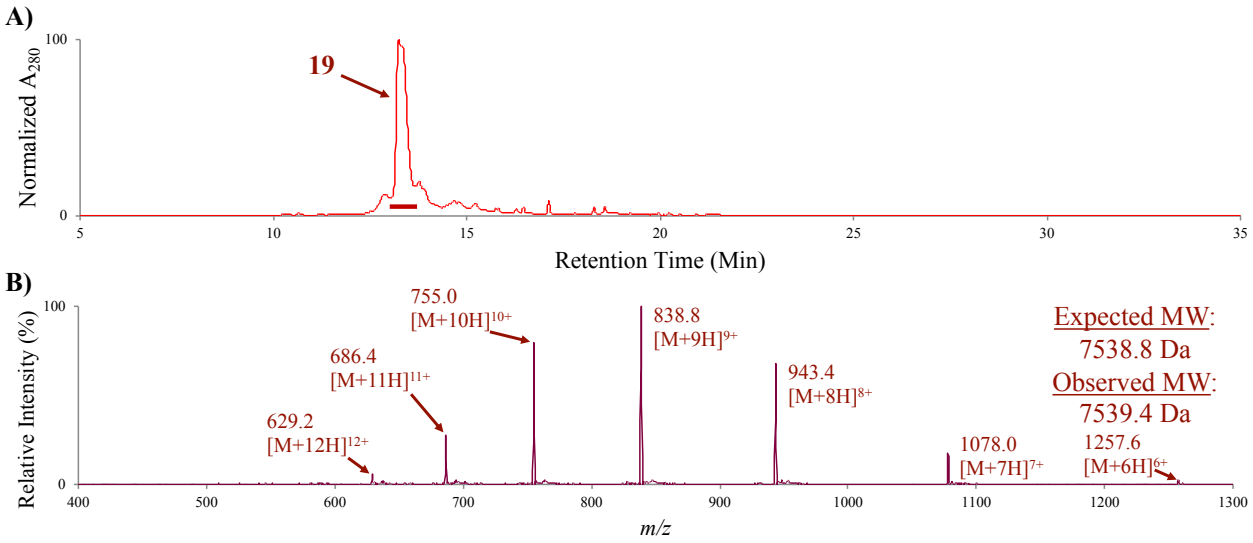


Figure S64: LC/MS analysis of the 360 min time point from the click-assisted native chemical ligation of 0.3 mM L32-Far-Post-Click peptide (**18**) shown in Figure S63. (A) LC/MS chromatogram. The double peaks of the ligated product (**19**) are expected, as the triazole has two regioisomers. (B) MS of **19** (dark red bar in part A). LC/MS method F was used for analysis.

SECTION 6: References

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