Site-specific N- and C-terminal labeling of a single polypeptide using sortases of different specificity

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Supporting Information

Contents:

I. Materials and Instrumentation

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. 4-Methylbenzylhydrylamine (MBHA) resin HL hydrochloride salt (100-200 mesh, 1.1 mmol/g), 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ala-OH, and Fmoc-ε-Ahx-OH were obtained from EMB Biosciences/Novabiochem. Rink amide resin (100-200 mesh, 0.7 mmol/g) was obtained from Advanced Chemtech. FITC isomer I was purchased from Sigma (F7250). α-Fmoc-ε-TMR-L-lysine was purchased from Invitrogen. Water used in biological procedures or as a reaction solvent was purified using a MilliQ purification system (Millipore). DriSolv® anhydrous CH_2Cl_2 and DriSolv® anhydrous MeOH were purchased from EMD Chemicals. Redistilled, anhydrous *N,N'*-diisopropylethylamine (DIPEA) was obtained from Sigma-Aldrich.

Mass Spectrometry. LC-ESI-MS analysis was performed using a Micromass LCT mass spectrometer (Micromass® MS Technologies, USA) and a Paradigm MG4 HPLC system equipped with a HTC PAL autosampler (Michrom BioResources, USA) and a Waters Symmetry 5 μm C8 column (2.1 x 50 mm, MeCN:H_2O (0.1% formic acid) gradient mobile phase, 150 μL/min).

HPLC/FPLC. HPLC purifications were achieved using an Agilent 1100 Series HPLC system equipped with a Waters Delta Pak 15 μm, 100 Å C18 column (7.8 x 300 mm, MeCN:H_2O gradient mobile phase, 3 mL/min) as indicated below. Size exclusion and cation exchange chromatography were performed on a Pharmacia AKTA Purifier system equipped with a HiLoad 16/60 Superdex 75 column (Amersham) or a Mono Q 5/50 GL column (Amersham), respectively.

UV-vis Spectrocopy. UV-vis spectroscopy was performed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

In-gel Fluorescence. Fluorescent gel images were obtained using a Typhoon 9200 Variable Mode Imager (GE Healthcare).

II. Synthesis and Characterization of FITC-LPRT-OMe (1), biotin-LPRT-OMe (2) and AA-TMR (3)

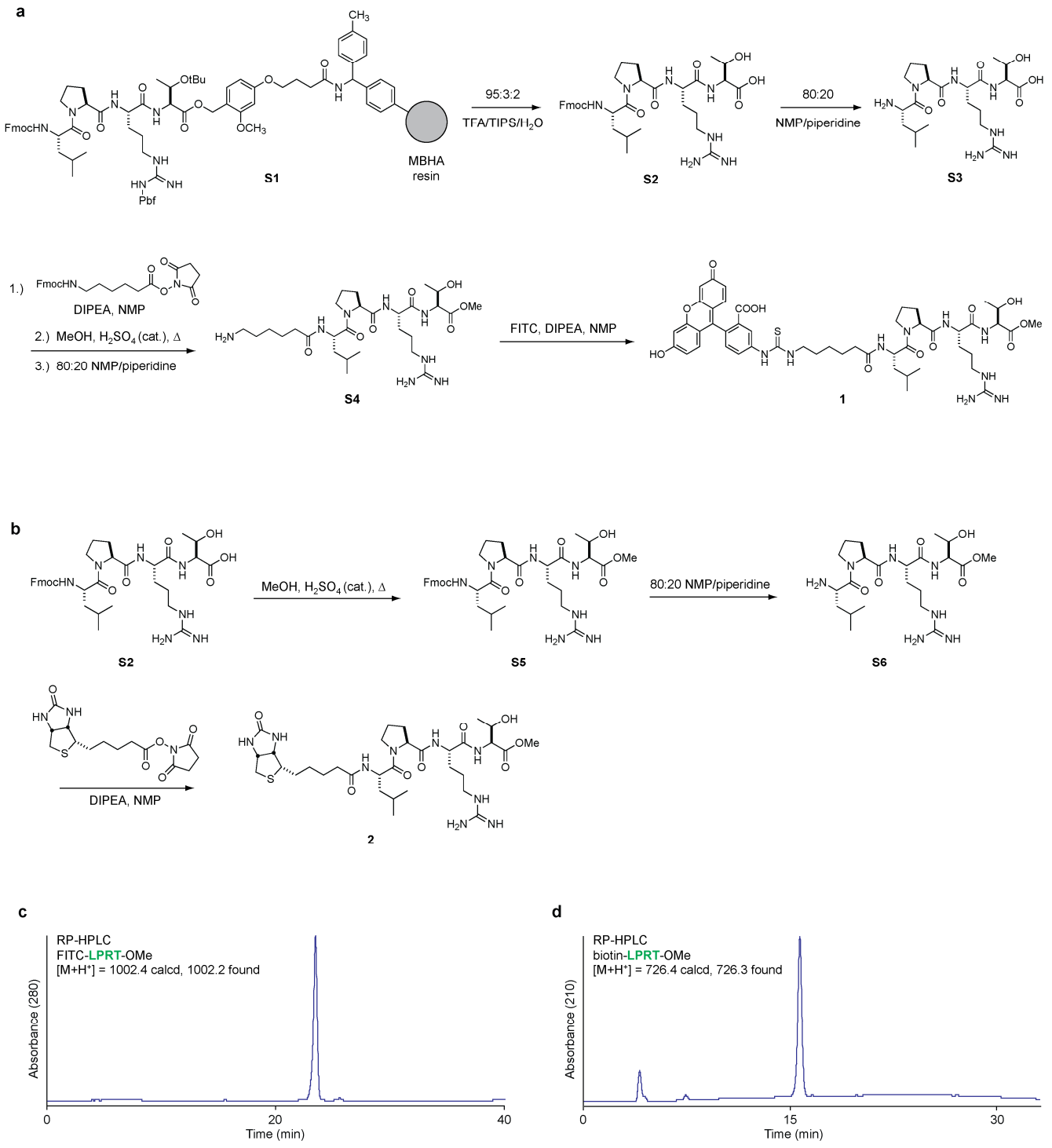

Figure S1. (**a**) Synthesis of FITC-LPRT-OMe (**1**) and biotin-LPRT-OMe (**2**). (**b**) RP-HPLC chromatogram (280 nm) for purified **1** and ESI-MS characterization. (**c**) RP-HPLC chromatogram (210 nm) for purified **2** and ESI-MS characterization.

Fmoc-LPRT-OH (S2). MBHA resin (1.05 g, 1.15 mmol) was first washed/swollen with 25 mL of NMP (3x, 3-5 min per wash). The resin was then treated with a solution of HMPB (690 mg, 2.87 mmol), PyBOP (1.49 g, 2.86 mmol), HOBt (387 mg, 2.86 mmol), and DIPEA (1.48 mL, 8.59 mmol) in 11.5 mL of NMP and incubated for 14 at RT with gentle agitation on a wrist action shaker. The resin was then washed with 25 mL of NMP (3x, 3-5 min per wash) followed by 25 mL of CH_2Cl_2 (3x, 10 min per wash). The resin was then dried and 370 mg of the dry resin was transferred to a new solid phase reaction vessel. The resin was washed with CH_2Cl_2 and then treated with a solution of Fmoc-Thr(tBu)-OH (477 mg, 1.20 mmol), DIC (186 μL, 1.20 mmol), and DMAP (14 mg, 0.11 mmol) in 5 mL of anhydrous CH_2Cl_2. The resin was incubated for 16 h at RT and then washed with 20 mL of CH_2Cl_2 (3x, 3-5 min per wash). The coupling of Fmoc-Thr(tBu)-OH was then repeated to achieve maximum resin loading. The resin was then washed with 20 mL of NMP (3x, 3-5 min per wash). Deprotection was achieved with 80:20 NMP/piperidine (20 mL) for 20 min at RT followed by washing with 20 mL of NMP (3x, 3-5 min per wash). The remaining amino acid building blocks (R,P,L) were then coupled as follows: Fmoc-protected amino acid (1.50 mmol, 5.0 equivalents relative to estimated resin loading), PyBOP (781 mg, 1.50 mmol), HOBt (203 mg, 1.50 mmol), and DIPEA (775 μL, 4.50 mmol) were dissolved in NMP to a final volume of 7.00 mL. This solution was mixed until all reagents had dissolved, and then added to the deprotected resin. Couplings were incubated for 12-24 h at RT. The resin was then washed with ~30 mL of NMP (3x, 3-5 min per wash). The extent of coupling was assessed by Kaiser test. In the event that the coupling was incomplete, the above procedure was repeated. Fmoc removal was then achieved by exposing the resin to 20 mL of 80:20 NMP/piperidine for 20 min at RT, followed by additional washing with ~30 mL of NMP (3x, 3-5 min per wash). Repeated cycles of amino acid coupling and Fmoc deprotection were then repeated to complete the synthesis of resin-bound intermediate **S1**. Resin **S1** was cleaved by treatment with ~5 mL of 95:3:2 TFA/TIPS/H_2O (5x, ~30 min each) and the combined cleavage solutions were concentrated. Crude **S2** was precipitated from cold diethyl ether and dried (184 mg, 87% yield based on estimated resin loading). The identity of **S2** was confirmed by ESI-MS ($[M+H^+]$ = 708.4 calcd, 708.4 obsd). **S2** was used without further purification.

H_2N-LPRT-OH (S3). Peptide **S2** (28 mg, 40 μmol) was dissolved in 160 μL of NMP and treated with 30 μL of piperidine. The reaction was incubated at room temperature for 20 min. Crude **S3** was precipitated from cold diethyl ether followed by washing of the resulting solid with three additional

portions of diethyl ether. The solid was then dried and used without further purification (8 mg, 41%). The identity of **S3** was confirmed by ESI-MS ([M+H$^+$] = 486.3 calcd, 486.1 obsd).

H_2N-Ahx-LPRT-OMe (S4). Intermediate **S3** (8.0 mg, 16 μmol) was combined with Fmoc-aminohexanoic acid NHS ester (Fmoc-Ahx-NHS)[1] (8.1 mg, 18 μmol), and DIPEA (15 μL, 86 μmol) in 172 μL of NMP. The reaction was incubated at room temperature for 19 h. The crude product was then precipitated from cold diethyl ether and the resulting solid washed with two additional portions of diethyl ether. The solid was then dissolved in 750 μL of anhydrous MeOH and treated with 5 μL of concentrated H_2SO_4. The reaction was incubated for 4 h at 50 ^oC. Formation of the desired methyl ester was verified by ESI-MS. The reaction was then diluted with 20 mL of H_2O and passed over a Waters Sep-Pak® Plus C18 Cartridge (cartridge was pre-equilibrated with 20 mL of 1:1 MeCN/H_2O followed by 20 mL of H_2O). The cartridge was washed with 20 mL of H_2O and the crude methyl ester was eluted with 10 mL of 1:1 MeCN/H_2O. This material was then concentrated and treated with 100 μL of 80:20 NMP/piperidine for 20 min at RT. Crude **S4** was then precipitated from cold diethyl ether, washed with two additional portions of diethyl ether, and dried under vacuum. **S4** was used without further purification (7 mg, 71% from **S3**). The identity of **S4** was confirmed by ESI-MS ([M+H$^+$] = 613.4 calcd, 613.3 obsd).

FITC-LPRT-OMe (1). Intermediate **S4** (7.0 mg, 11 μmol) was combined with FITC isomer I (4.9 mg, 13 μmol), and DIPEA (15 μL, 86 μmol) in 120 μL of NMP. The reaction was incubated at room temperature for 2 h. Crude **1** was then precipitated from cold diethyl ether and the resulting solid washed with two additional portions of diethyl ether. The material was then purified by RP-HPLC [Waters C18 column, MeCN:H_2O gradient mobile phase, 3 mL/min, 5% MeCN (0-2 min), 5% MeCN $\rightarrow$ 40% MeCN (2-25 min)] to yield **1** (3.8 mg, 35%). The identity and purity of **1** was confirmed by RP-HPLC and ESI-MS (**Figure S1b**). A 10 mM stock solution of **1** in DMSO was used in all transpeptidation experiments.

Fmoc-LPRT-OMe (S5). Peptide **S2** (35 mg, 49 μmol) was dissolved in 750 μL of anhydrous MeOH and treated with 5 μL of concentrated H_2SO_4. The reaction was incubated for 3 h at 55 ^oC followed by an additional 15 h at RT. The reaction was then diluted with 20 mL of H_2O and passed over a Waters Sep-Pak® Plus C18 Cartridge (cartridge was pre-equilibrated with 20 mL of 1:1 MeCN/H_2O followed by

20 mL of H_2O). The cartridge was washed with 20 mL of H_2O and then crude **S5** was eluted with 10 mL of 1:1 MeCN/H_2O. **S5** was then concentrated and used without further purification (24 mg, 68%). The identity of **S5** was confirmed by ESI-MS ([M+H$^+$] = 722.4 calcd, 722.5 obsd).

H_2N-LPRT-OMe (S6). Methyl ester **S5** (23 mg, 32 μmol) was dissolved in 125 μL of NMP and treated with 30 μL of piperidine. The reaction was incubated at room temperature for 20 min. Crude **S6** was precipitated from cold diethyl ether followed by washing of the resulting solid with three additional portions of diethyl ether. The solid was then dried and used without further purification (16 mg, quantitative). The identity of **S6** was confirmed by ESI-MS ([M+H$^+$] = 500.3 calcd, 500.2 obsd).

Biotin-LPRT-OMe (2). Intermediate **S6** (5.0 mg, 10 μmol) was combined with biotin-NHS[2] (3.8 mg, 11 μmol), and DIPEA (10 μL, 58 μmol) in 105 μL of NMP. The reaction was incubated at room temperature for 18 h. The material was then purified by RP-HPLC [Waters C18 column, MeCN:H_2O gradient mobile phase, 3 mL/min, 5% MeCN (0-2 min), 5% MeCN → 60% MeCN (2-30 min)] to yield **2** (3 mg, 41%). The identity and purity of **2** was confirmed by RP-HPLC and ESI-MS (**Figure S1d**). A 10 mM stock solution of **2** in DMSO was used in all transpeptidation experiments.

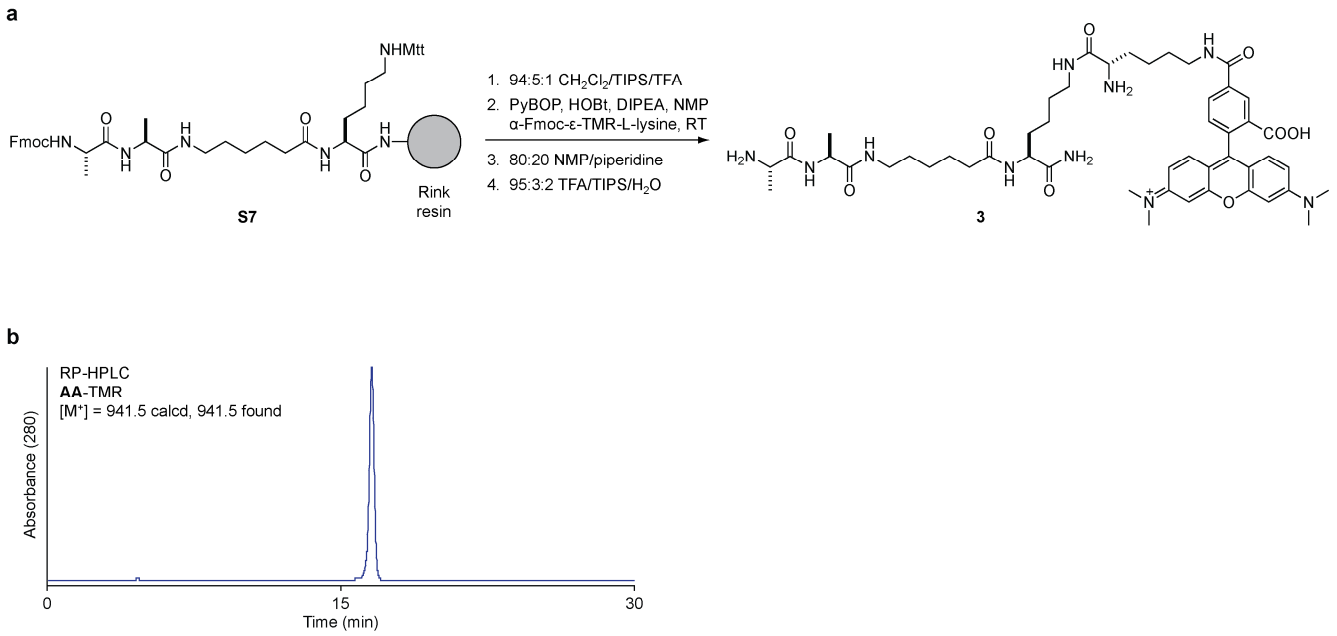

Figure S5. (**a**) Synthesis of AA-TMR (**3**). (**b**) RP-HPLC chromatogram (280 nm) for purified **3** and ESI-MS characterization.

AA-TMR (3). Resin bound intermediate **S7** was synthesized on Rink amide resin using standard Fmoc synthesis. Couplings were performed using 5 equivalents (relative to estimated resin loading) of the suitably protected Fmoc amino building block, 5 equivalents of PyBOP, 5 equivalents of HOBt, and 15 equivalents of DIPEA in NMP (final concentration of Fmoc amino acid was ~170 mM). Couplings were run for 5-72 h at room temperature with gentle agitation on a wrist action shaker. Fmoc deprotection was achieved with 80:20 NMP/piperidine for 20 min at room temperature. The resin was washed with NMP between each transformation. Following completion of **S5**, the resin was washed with NMP (4x, 3-5 min per wash) and CH_2Cl_2 (5x, 3-5 min per wash). The resin was then dried. Dry **S5** resin (43 mg, 21 μmol, 0.5 mmol/g estimated resin loading) was then transferred to a 3.0 mL fritted polypropylene syringe equipped with a hypodermic needle. The 4-methyltrityl (Mtt) protecting group was removed by treatment with 2.5 mL of 94:5:1 CH_2Cl_2/TIPS/TFA at room temperature (5x, 5 min each) followed by washing with 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash) and 2.5 mL of NMP (3x, 3-5 min per wash). The resin was then treated with a solution of α-Fmoc-ε-TMR-L-lysine (25 mg, 32 μmol), PyBOP (17 mg, 33 μmol), HOBt (9 mg, 70 μmol), and DIPEA (16.5 μL, 95.8 μmol) in 1.0 mL of NMP. The reaction was incubated at RT for 20 h followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash). Fmoc removal was achieved by treatment with 2.5 mL of 80:20 NMP/piperidine for 20 min at RT followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash) and 2.5 mL of CH_2Cl_2 (3x,

3-5 min per wash). The peptide was cleaved from the resin with 2.5 mL of 95:3:2 TFA/TIPS/H_2O (5x, ~15 min each) and the combined cleavage solutions were concentrated *in vacuo*. Crude **3** was precipitated from cold diethyl ether, and then purified by RP-HPLC [Waters C18 column, MeCN:H_2O gradient mobile phase containing 0.1% TFA, 3 mL/min, 5% MeCN (0-2 min), 5% MeCN → 60% MeCN (2-30 min)]. The identity and purity of **3** (8 mg, 40%) was confirmed by RP-HPLC and ESI-MS (**Figure S5b**). A 100 mM stock solution of **3** in water was used for transpeptidation experiments.

III. Protein Cloning and Expression

$SrtA_{strep}$. The expression plasmid for $SrtA_{strep}$ (residues 82-249) including an N-terminal His_6 tag has been described.[3] The construct was transformed into *E. coli* BL-21. Cells were grown in 2 L of sterile LB containing kanamycin (30 μg/mL) to an optical density of ~0.7 at 600 nm. Cells were induced with IPTG (1 mM) for 3 h at 37 ^oC. Cells were harvested by centrifugation and the pellet was stored overnight at -20 ^oC. The pellet was thawed and resuspended in 70 mL of 50 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 10% glycerol. Cells were then treated with 300 μL of DNAse I (10 mg/mL in PBS), 500 μL of lysozyme (50 mg/mL in PBS), and 10 μL of $MgCl_2$ (1 M in PBS). The lysis reaction was incubated for 1 h at 4 ^oC. The cells were then sonicated and centrifuged to remove insoluble material. The clarified lysate was then applied to a Ni-NTA column consisting of 5.0 mL of commercial Ni-NTA slurry (Qiagen) equilibrated with 50 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole, and 10% glycerol. The column was washed with 80 mL of 50 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole, and 10% glycerol. Protein was eluted with five 5 mL portions of 50 mM Tris pH 8.0, 150 mM NaCl, 300 mM imidazole, and 10% glycerol. Fractions containing $SrtA_{strep}$ were pooled and further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 column (Amersham), eluting with 20 mM Tris pH 8.0, 150 mM NaCl at a flow rate of 1 mL/min. Fractions containing $SrtA_{strep}$ were pooled and subjected to a second round of Ni-affinity chromatography. Purified $SrtA_{strep}$ was then dialyzed against 50 mM Tris pH 8.0, 150 mM NaCl, and 10% glycerol. These solutions were stored at -80 ^oC until further use. Protein concentration was estimated by Bradford assay.

$SrtA_{staph}$. Recombinant $SrtA_{staph}$ (residues 26-206) containing an N-terminal His_6 tag was produced in *E. coli* as previously described.[4] $SrtA_{staph}$ does not contain an N-terminal glycine residue (retains initiator methionine). Purified $SrtA_{staph}$ was stored in 10% (w/v) glycerol, 50 mM Tris pH 8.0, 150 NaCl at -80 °C until further use. Protein concentration was estimated by Bradford assay.

Δ59-$SrtA_{staph}$. Recombinant Δ59-$SrtA_{staph}$ (residues 60-206) containing an N-terminal His_6 tag was cloned into pET28a+. Δ59-$SrtA_{staph}$ does not contain an N-terminal glycine residue (retains initiator methionine). Expression of Δ59-$SrtA_{staph}$ was achieved following the protocol described above for $SrtA_{strep}$. Purification by size exclusion chromatography was not necessary because Δ59-$SrtA_{staph}$ was sufficiently pure following Ni-affinity chromatography.

CtxB. The template for construction of G_1-CtxB, G_3-CtxB, G_5-CtxB, and AG_4-CtxB consisted of the B-subunit of cholera toxin fused at its N terminus to the signal peptide sequence of *E. coli* heat labile enterotoxin LTIIb.[5] This targets the expressed protein to the periplasm where the signal peptide is removed. Glycine and/or alanine residues were inserted between the signal sequence and CtxB via Quickchange® II Site-Directed Mutagenesis (Stratagene). Plasmids were transformed into *E. coli* BL-21. Cells were grown in 1 L of sterile LB containing chloroamphenicol (34 μg/mL) to an optical density of ~0.5-1.0 at 600 nm. Cells were induced with arabinose (0.25% w/v) for 3 h at 37 ^oC. Cells were harvested by centrifugation and the pellet was stored overnight at -20 ^oC. The pellet was thawed and resuspended in 30 mL of 50 mM Tris pH 8.0 and 300 mM NaCl. This suspension was then treated with 3 mL of polymixin B solution (5 mg/mL freshly made in water). This was mixture was gently stirred at room temperature for 1 h and then centrifuged. The clarified lysate was treated with 2.5 mL of Ni-NTA slurry (Qiagen). CtxB has a naturally affinity for Ni-NTA although it does not possess a His_6 tag. The Ni-NTA mixture was incubated at 4 ^oC for 1 h and then poured into a fritted plastic column (Bio-Rad). The resin was washed with 40 mL of 50 mM Tris pH 8.0 and 300 mM NaCl. Protein was eluted with two 10 mL portions of 50 mM Tris pH 8.0, 300 mM NaCl, and 300 mM imidazole. Purified CtxB was buffer exchanged into 20 mM Tris pH 8.0 and 150 mM NaCl. Solutions were stored at 4 ^oC. Protein concentration was estimated by Bradford assay.

G_5-eGFP and dual labeling eGFP substrate. G_5-eGFP (containing a C-terminal His_6 tag) and the eGFP dual labeling substrate (containing an N-terminal thrombin cleavage site and a C-terminal LPETG

motif followed by a His_6 tag) and were prepared in pET28a+ (Novagen) using a Quickchange® II Site-Directed Mutagenesis Kit (Stratagene). The template plasmid used for mutagenesis has been described.[6] Plasmids were then transformed into *E. coli* BL-21. In a typical experiment, cells were grown in sterile LB containing kanamycin (30 μg/mL) to an optical density of ~0.6-0.9 at 600 nm. Cells were induced with IPTG (1 mM) for 3 h at 37 ^{o}C. Cells were harvested by centrifugation and the pellet was stored overnight at -20 ^{o}C. The pellet was thawed and resuspended in 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40. The cell suspension was then lysed by French press and centrifuged. The clarified lysate was then applied to a Ni-NTA column consisting of 5.0 mL of commercial Ni-NTA slurry (Qiagen) equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40. The column was washed with 40 mL of 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40, followed by 40 mL of 20 mM Tris pH 8.0, 150 mM NaCl, and 20 mM imidazole. Protein was eluted with 20 mM Tris pH 8.0, 150 mM NaCl, and 300 mM imidazole until the characteristic green color was fully removed from the column. This material was concentrated and further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 column (Amersham), eluting with 20 mM Tris pH 8.0, 150 mM NaCl at a flow rate of 1 mL/min. Fractions containing eGFP were pooled and subjected to a second round of Ni-affinity chromatography. Purified eGFP was then buffer exchanged into 20 mM Tris pH 8.0, 150 mM NaCl using a PD-10 Sephadex™ column (GE Healthcare), concentrated, and treated with glycerol (10% v/v final concentration). These solutions were stored at -80 ^{o}C until further use. Protein concentration was estimated by UV-vis spectroscopy using the absorbance of eGFP at 488 nm (extinction coefficient 55,900 $M^{-1}cm^{-1}$).[7]

UCHL3 and dual labeling UCHL3 substrate. UCHL3 containing a single N-terminal glycine residue was produced in *E. coli* as described previously.[8] This construct (in pET28a+, Novagen) was then used to prepare the dual labeling UCHL3 substrate. Synthetic 5′-phosphorylated oligonucleotide duplexes containing appropriate sticky ends were designed to achieve insertion of an N-terminal thrombin site and a C-terminal LPETG sequence separated from UCHL3 by a GGGGSGGGGS spacer in two sequential cloning steps. Duplexes were annealed before ligation into the parent vector. The C-terminal insertion was performed first using the PstI and XhoI restriction sites. The result of using the XhoI site was the addition of a His_6-tag after the LPETG sequence. The N-terminal insertion was then achieved using the XbaI and NdeI restriction sites. This plasmid was then transformed into *E. coli* BL21. Cells were grown in sterile LB containing kanamycin (30 μg/mL) to an optical density of ~0.6-0.9 at 600 nm.

Cells were induced with IPTG (1 mM) for 3 h at 37 ^{o}C. Bacteria were then harvested by centrifugation and the pellet was stored overnight at -20 ^{o}C. The pellet was thawed and resuspended in 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40. The cell suspension was then lysed by French press and centrifuged. The clarified lysate was then applied to a Ni-NTA column consisting of 5.0 mL of commercial Ni-NTA slurry (Qiagen) equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40. The column was washed with 40 mL of 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole and 1% NP-40, followed by 40 mL of 20 mM Tris pH 8.0, 150 mM NaCl, and 20 mM imidazole. Protein was eluted with 20 mM Tris pH 8.0, 150 mM NaCl, and 300 mM imidazole. This material was then purified by anion-exchange chromatography on a Mono Q 5/50 GL column (Amersham) [Buffer A (50 mM Tris pH 7.5, 5 mM DTT, 0.5 mM EDTA), Buffer B (50 mM Tris pH 7.5, 5 mM DTT, 0.5 mM EDTA, 500 mM NaCl), 1.5 mL/min, gradient: 100% Buffer A (0-15 mL), 0% Buffer B $\rightarrow$ 50% Buffer B (15-45 mL), 50% Buffer B (45-50 mL)]. Fractions containing UCHL3 were pooled and further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 column (Amersham), eluting with 20 mM Tris pH 8.0, 150 mM NaCl at a flow rate of 1 mL/min. Fractions containing UCHL3 were pooled and subjected to a final purification step by anion-exchange chromatography on a Mono Q 5/50 GL column (Amersham) [Buffer A (50 mM phosphate pH 6.0), Buffer B (50 mM phosphate pH 6.0, 500 mM NaCl), 1.5 mL/min, gradient: 100% Buffer A (0-15 mL), 0% Buffer B $\rightarrow$ 50% Buffer B (15-45 mL), 50% Buffer B (45-50 mL)]. The dual labeling UCHL3 substrate was buffer exchanged into 20 mM Tris pH 8.0, 150 NaCl and protein concentration was estimated by Bradford assay.

IV. Sortase-Mediated Labeling of Protein Substrates

N-terminal labeling. N-terminal transpeptidation reactions were performed by combining the necessary proteins/reagents at the specified concentrations in the presence of $SrtA_{staph}$ or $\Delta59\text{-}SrtA_{staph}$ in sortase reaction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$) and incubating at 37 °C for the times indicated. Reactions were either diluted with 2x reducing Laemmli sample buffer for SDS-PAGE analysis or diluted with water (~50 fold) for ESI-MS analysis. Gels were visualized by staining with coomassie blue. Fluorescence was visualized on a Typhoon 9200 Imager (GE Healthcare). For detection of biotinylation, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with a streptavidin-horseradish peroxidase conjugate (GE Healthcare) and visualized by chemiluminescence. ESI-MS was performed on a Micromass LCT mass spectrometer (Micromass® MS Technologies, USA) and a Paradigm MG4 HPLC system equipped with a HTC PAL autosampler (Michrom BioResources, USA) and a Waters Symmetry 5 μm C8 column (2.1 x 50 mm, MeCN:H_2O (0.1% formic acid) gradient mobile phase, 150 μL/min).

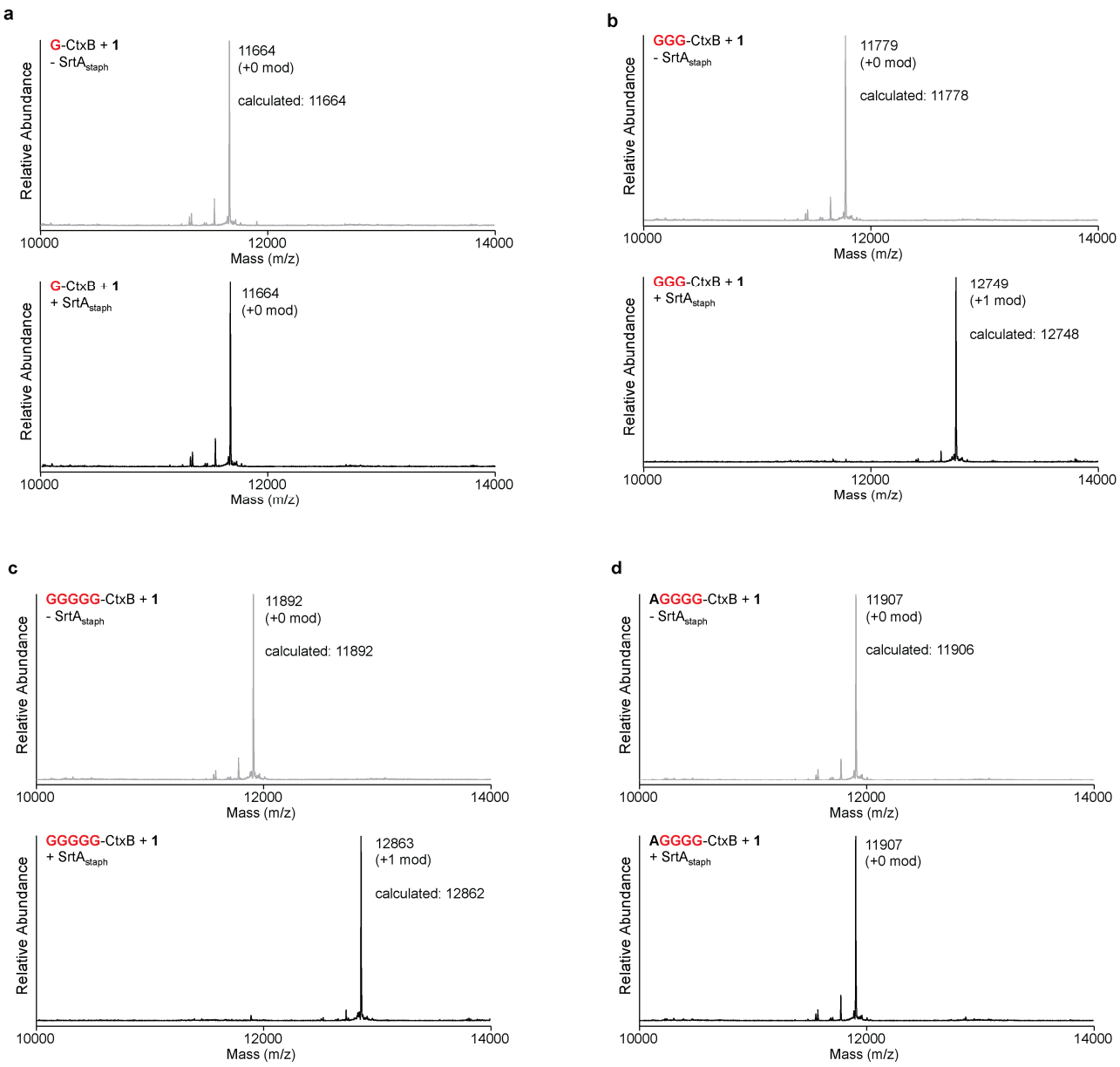

Figure S2. Reconstructed ESI-MS spectra for N-terminal labeling reactions on (**a**) G_1-CtxB, (**b**) G_3-CtxB, (**c**) G_5-CtxB, and (**d**) AG_4-CtxB substrates. Conditions: 50 μM CtxB, 50 μM $SrtA_{staph}$, 500 μM **1**, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 2 h at 37 ^{o}C.

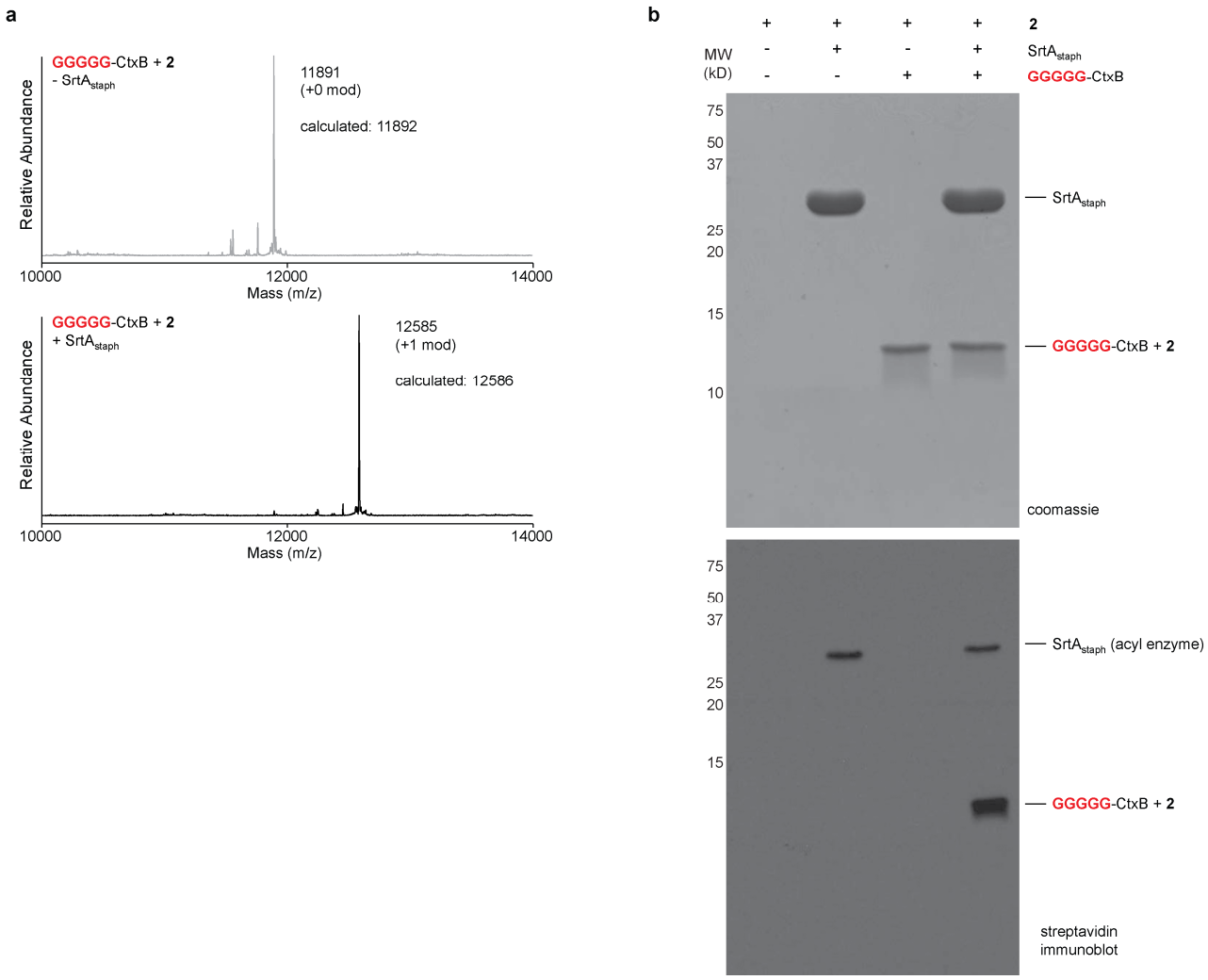

Figure S3. Site-specific N-terminal biotinylation of G_5-CtxB. Conditions: 33 μM G_5-CtxB, 50 μM $SrtA_{staph}$, 500 μM **2**, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 2 h at 37 °C. (**a**) ESI-MS characterization of biotinylation. (**b**) Verification of biotinylation by streptavidin immunblot.

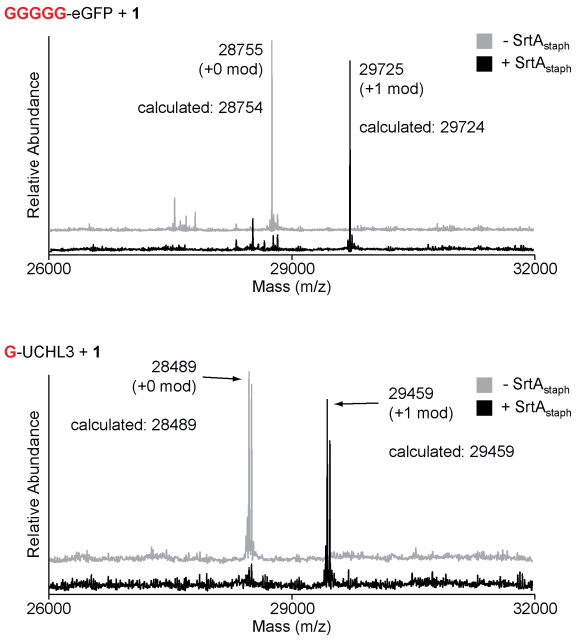

Figure S4. Additional substrates for N-terminal labeling. (**a**) ESI-MS spectra for G_5-eGFP labeling. Conditions: 50 μM G_5-eGFP, 50 μM $SrtA_{staph}$, 500 μM **1**, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 2 h at 37 °C. (**b**) ESI-MS spectra for G_1-UCHL3 labeling. Conditions: 50 μM G_1-UCHL3, 50 μM $SrtA_{staph}$, 500 μM **1**, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 2 h at 37 °C.

Dual labeling of eGFP

Immediately prior to starting the dual labeling sequence, the eGFP stock was thawed and again purified by affinity chromatography over commercial Ni-NTA resin. After binding eGFP to the resin, the column was washed with 20 mM Tris pH 8.0, 150 mM NaCl, and 20 mM imidazole. The protein was eluted with 20 mM Tris pH 8.0, 150 mM NaCl, and 300 mM imidazole. This material was buffer exchanged into 20 mM Tris pH 8.0, 150 mM NaCl using a NAP™ 5 Sephadex™ column (GE Healthcare) and concentrated. The concentration was estimated to be 84 μM by UV-vis spectroscopy using eGFP absorbance at 488 nm (extinction coefficient 55,900 $M^{-1}cm^{-1}$).[7]

*C-terminal modification of eGFP with **3** and $SrtA_{strep}$.* 400 μL of the freshly purified eGFP solution was then treated with $SrtA_{strep}$ (87 μL of a 140 μM stock solution) and **3** (4.9 μL of a 100 mM stock solution) [**Note:** $SrtA_{strep}$ does not require Ca^{2+} for activity].[3] The reaction was incubated for 7 h at 37 °C. ESI-MS analysis of the crude reaction mixture revealed excellent conversion to the desired product (Supporting Figure S6a). The reaction was then treated with [2-(trimethylammonium)ethly] methane

thiosulfonate bromide (MTSET) (2.5 μL of a 500 mM solution in 1:1 DMSO/H_2O) for 10 min at room temperature to quench $SrtA_{strep}$. The entire reaction was then diluted with 5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. This solution was then passed over a 1.5 mL column of Ni-NTA that been equilibrated with 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. The column was then washed with 1.5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. His_6-tagged $SrtA_{strep}$ was bound by Ni-NTA while the eGFP product (which lost its His_6 tag during the course of transpeptidation) was not retained. The eGFP solution was then concentrated and passed over a PD-10 Sephadex™ desalting column (equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl) to remove excess **3**. This material was concentrated to ~800 μL and subjected to thrombin cleavage.

Thrombin cleavage of eGFP. All 800 uL of the solution described above was combined with 100 μL of 10x cleavage buffer and 100 μL of thrombin agarose beads (from Thrombin CleanCleave™ Kit, Sigma). This mixture was incubated for 1 h at 37 °C, and then checked by ESI-MS to ensure quantitative cleavage (Supporting Figure S6a). The reaction was then filtered to remove the thrombin beads.

*N-terminal labeling of eGFP with **1** and Δ59-$SrtA_{staph}$.* 389 μL of the thrombin cleaved material was combined with **1** (25 μL of a 10 mM DMSO solution), Δ59-$SrtA_{staph}$ (36 μL of a 700 μM stock solution), and 10x sortase reaction buffer (50 μL of 500 mM Tris pH 8.0, 1.5 M NaCl, 100 mM $CaCl_2$). The reaction was incubated for 75 min at 37 °C. ESI-MS of the crude reaction mixture showed clean formation of the dual labeled product as the major reaction product (Supporting Figure S6a). The reaction was then diluted with 5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. This solution was passed over a 1.5 mL column of Ni-NTA that been equilibrated with 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole in order to remove His_6-tagged Δ59-$SrtA_{staph}$. 2.5 mL of this eluate was then passed over a PD-10 Sephadex™ desalting column (equilibrated with 20 mM Tris pH 8.0). This material was then purified by anion-exchange chromatography on a Mono Q 5/50 GL column (Amersham) [Buffer A (20 mM Tris pH 8.0), Buffer B (20 mM Tris pH 8.0, 1 M NaCl), 1.5 mL/min, gradient: 100% Buffer A (0-15 mL), 0% Buffer B → 50% Buffer B (15-45 mL), 50% Buffer B (45-50 mL)]. Fractions containing dual labeled eGFP were pooled and analyzed by SDS-PAGE and ESI-MS. Coomassie stained gels were imaged using a CanoScan 8600F scanner. Protein purity was estimated from these images using ImageJ 1.42q densitometry software.

Dual labeling of UCHL3

*C-terminal modification of UCHL3 with **3** and $SrtA_{strep}$.* UCHL3 (350 μL of a 65 μM stock solution) was treated with $SrtA_{strep}$ (76 μL of a 140 μM stock solution) and **3** (4.3 μL of a 100 mM stock solution) [**Note:** $SrtA_{strep}$ does not require Ca^{2+} for activity].[3] The reaction was incubated for 15 h at 37 °C. ESI-MS analysis of the crude reaction mixture revealed excellent conversion to the desired product (Supporting Figure S6b). The entire reaction was then diluted with 5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. This solution was then treated with [2-(trimethylammonium)ethly] methane thiosulfonate bromide (MTSET) (5.0 μL of a 500 mM solution in 1:1 DMSO/H_2O) for 10 min at room temperature to quench $SrtA_{strep}$. [**Note:** UCHL3 contains an active site cysteine residue and is therefore modified by MTSET. The resulting modification is disulfide linked, and is easily removed by treatment with DTT following completion of the dual labeling procedure]. The diluted reaction solution was then passed over a 1.5 mL column of Ni-NTA that been equilibrated with 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. The column was then washed with 1.5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. The UCHL3 solution was then concentrated and passed over a PD-10 Sephadex™ desalting column (equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl) to remove excess **3**. This material was concentrated to 1 mL and subjected to thrombin cleavage.

Thrombin cleavage of UCHL3. All 1 mL of the solution described above was combined with 100 μL of 10x cleavage buffer and 100 μL of thrombin agarose beads (from Thrombin CleanCleave™ Kit, Sigma). This mixture was incubated for 1 h at 37 °C, and then checked by ESI-MS to ensure quantitative cleavage (Supporting Figure S6b). The reaction was then filtered to remove the thrombin beads.

*N-terminal labeling of UCHL3 with **1** and Δ59-$SrtA_{staph}$.* 778 μL of the thrombin cleaved material was combined with **1** (50 μL of a 10 mM DMSO solution), Δ59-$SrtA_{staph}$ (72 μL of a 700 μM stock solution), and 10x sortase reaction buffer (100 μL of 500 mM Tris pH 8.0, 1.5 M NaCl, 100 mM $CaCl_2$). The reaction was incubated for 60 min at 37 °C. ESI-MS of the crude reaction mixture showed clean formation of the dual labeled product as the major reaction product (Supporting Figure S6b). The reaction was then diluted with 5 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. This solution was passed over a 1.5 mL column of Ni-NTA that been equilibrated with 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole in order to remove His_6-tagged Δ59-$SrtA_{staph}$. The column was

then washed with 2.0 mL of 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole. The eluate was then desalted using PD-10 Sephadex^{TM} columns (equilibrated with 20 mM Tris pH 8.0). This material was then purified by anion-exchange chromatography on a Mono Q 5/50 GL column (Amersham) [Buffer A (20 mM Tris pH 8.0), Buffer B (20 mM Tris pH 8.0, 1 M NaCl), 1.5 mL/min, gradient: 100% Buffer A (0-15 mL), 0% Buffer B $\rightarrow$ 50% Buffer B (15-45 mL), 50% Buffer B (45-50 mL)]. Fractions containing dual labeled UCHL3 were pooled and analyzed by SDS-PAGE and ESI-MS. Prior to ESI-MS, dual labeled UCHL3 was treated with 10 mM DTT for 10 min at RT to remove the MTSET modification on the active site cysteine residue. Coomassie stained gels were imaged using a CanoScan 8600F scanner. Protein purity was estimated from these images using ImageJ 1.42q densitometry software.

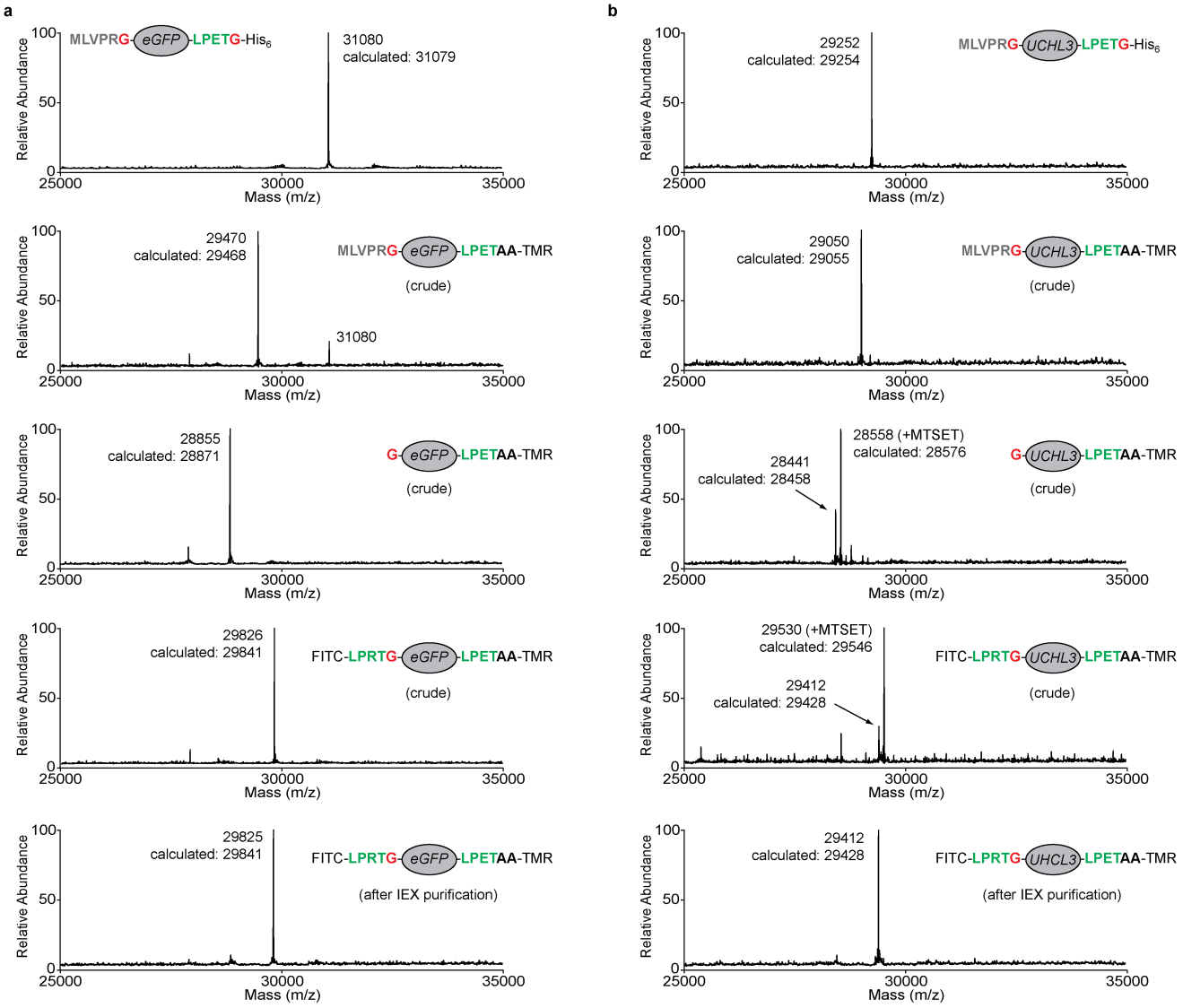

Figure S6. Site-specific labeling at the N and C termini of eGFP and UCHL3. (**a**) ESI-MS spectra for all intermediates generated during the double labeling procedure. From top to bottom, this includes the eGFP starting material (m/z = 31080 Da), the intermediate formed after C-terminal modification with **3** mediated by $SrtA_{strep}$ (m/z = 29470 Da), the product of thrombin cleavage (m/z = 28855 Da), crude dual labeled eGFP (m/z = 29726 Da), and dual labeled eGFP after anion exchange chromatography (m/z = 29725 Da). (**b**) ESI-MS spectra for all intermediates generated during double labeling of UCHL3. From top to bottom, this includes the UCHL3 starting material (m/z = 29252 Da), the intermediate formed after C-terminal modification with **3** mediated by $SrtA_{strep}$ (m/z = 29050 Da), the product of thrombin cleavage (m/z = 28458 Da), crude dual labeled UCHL3 (m/z = 29412 Da), and dual labeled UCHL3 after anion exchange chromatography (m/z = 29412 Da). The MTSET reagent used to quench $SrtA_{strep}$ also modifies the active site cysteine of UCHL3 creating an extra +118 Da signal in the mass spectrum. This modification is easily removed from the final product by brief treatment with DTT.

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