Supplementary Information

Upstream proteolysis by Ste24 does not require a C-terminal methyl ester as revealed using 33-residue a-factor precursor peptide substrates synthesized via epimerization-free methods

Taysir Bader,[1] Shanica Brown,[2] Christine Hrycyna[2,3] and Mark D. Distefano[1]

[1]Department of Chemistry, University of Minnesota, Minneapolis, MN, USA

[2]Department of Chemistry, Purdue University, West Lafayette, IN, USA

[3]Department of Chemistry and Biochemistry, University of California, San Diego, San Diego, CA, USA

Correspondence

Christine Hrycyna, [3]Department of Chemistry and Biochemistry, University of California, San Diego, San Diego, CA, USA

Email: chrycyna@ucsd.edu

Mark D. Distefano, [1]Department of Chemistry, University of Minnesota, Minneapolis, MN, USA

Email: diste001@umn.edu

Table of Contents

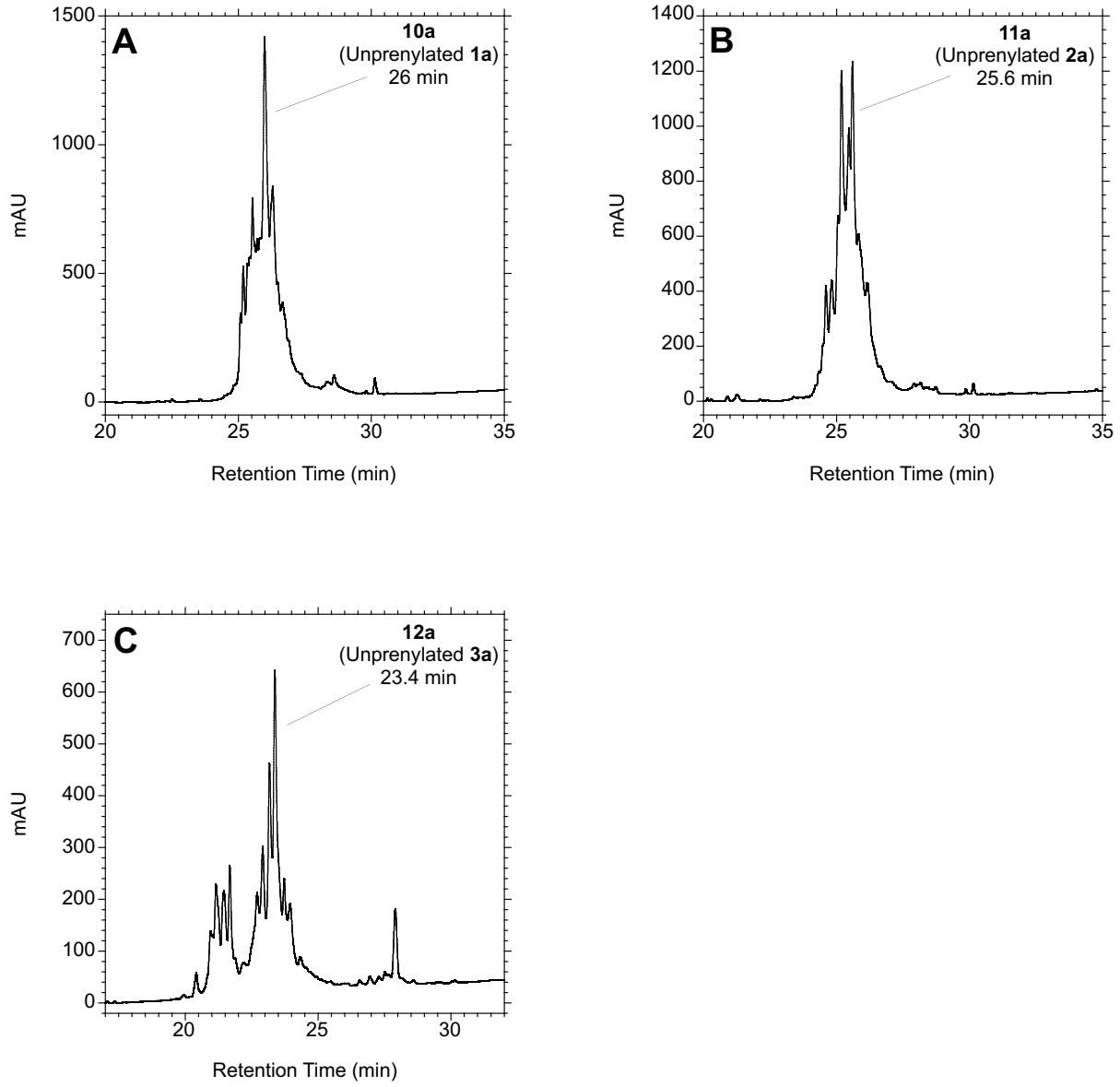

Fig. S1. LC-MS chromatograms of crude 33mer peptides after resin cleavage and global deprotection. LC-MS chromatograms highlighting the crude purity of the unprenylated precursors of the three 33mer analogues used in this study prior to prenylation. (**A**) C-terminal methyl ester peptide before farnesylation (**10a**). This peptide is the precursor in the synthesis of **1a**. (**B**) C-terminal carboxylic acid peptide before farnesylation (**11a**). This peptide is the precursor in the synthesis of **2a**. (**C**) C-terminal amide peptide before farnesylation (**12a**). This peptide is the precursor in the synthesis of **3a**. UV absorbance was monitored at 220 nm. The gradient used was as follows: 1-10 min, hold at 1% B. 10-35 min, gradient to 100% B. 35-40, hold at 100% B (column wash). 40-41, ramp to 1% B. 41-51, hold at 1% B (column equilibration).

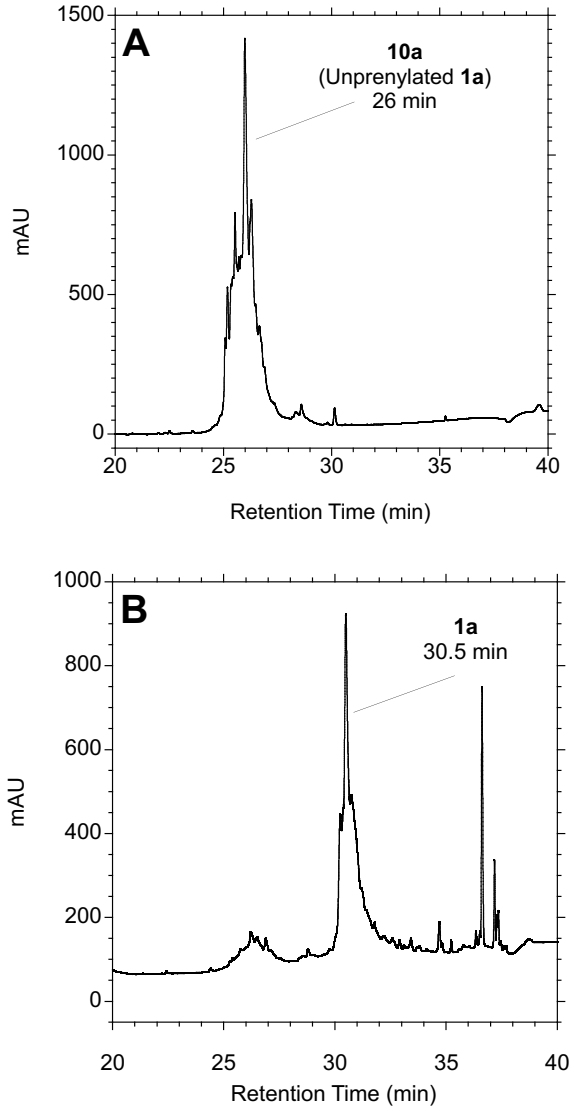

Fig. S2. Prenylation of peptide (**1a**) using $Zn(OAc)_2$ mediated reaction and farnesyl bromide. Zn(OAc)2 coordinates with the thiol at pH 5.0, modulating its nucleophilicity to be able to react with farnesyl bromide and displace the halide. (A) Shows LC-MS chromatogram of the precursor to peptide (1a) with free thiol. (B) shows LC-MS chromatogram of the peptide after prenylation, affording peptide (1a). Characteristic retention time shift and MS confirmed the correct product. UV absorbance was monitored at 220 nm. The gradient used was as follows: The gradient used was as follows: 1-10 min, hold at 1% B. 10-35 min, gradient to 100% B. 35-40, hold at 100% B (column wash). 40-41, ramp to 1% B. 41-51, hold at 1% B (column equilibration). The increased hold time at the beginning of the method was essential to remove the DMF fully, enhancing the resolution.

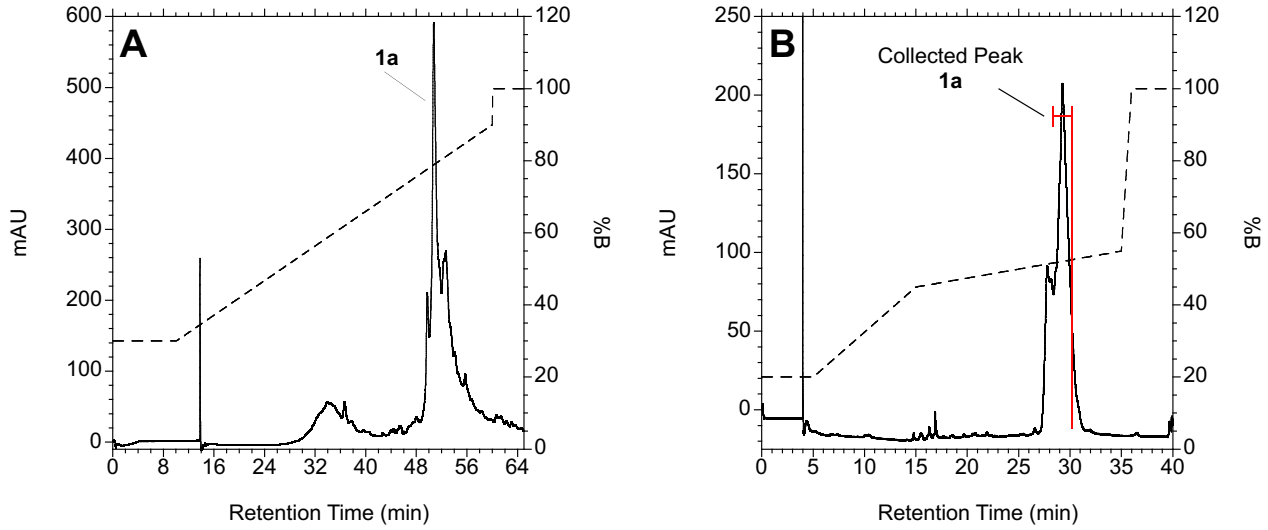

Fig. S3. Two-stage purification of peptide (**1a**). (**A**) Prep purification of peptide (**1a**). This initial stage helped concentrate the desired peptide. 73% purity was obtained afterwards. (**B**) Semi-prep purification using targeted gradient. This stage helped bring the purity to > 95%. This the shallow gradient helped improve the separation between closely eluting truncated peptide materials. UV absorbance was monitored at 280 nm, which helped improve the observed resolution.

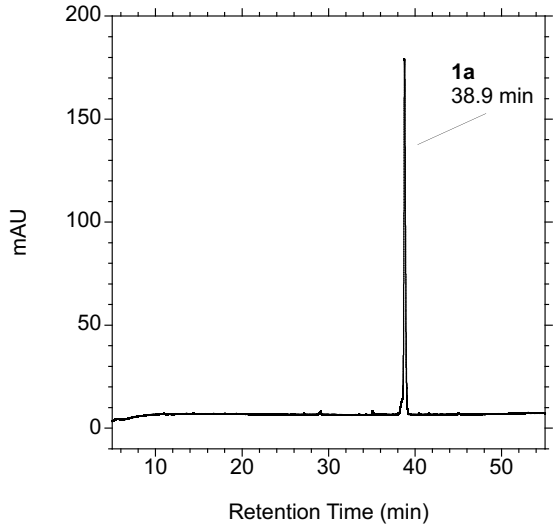

Fig. S4. HPLC chromatogram of peptide **1a** after two-stage HPLC purification. UV absorbance was monitored at 220 nm. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

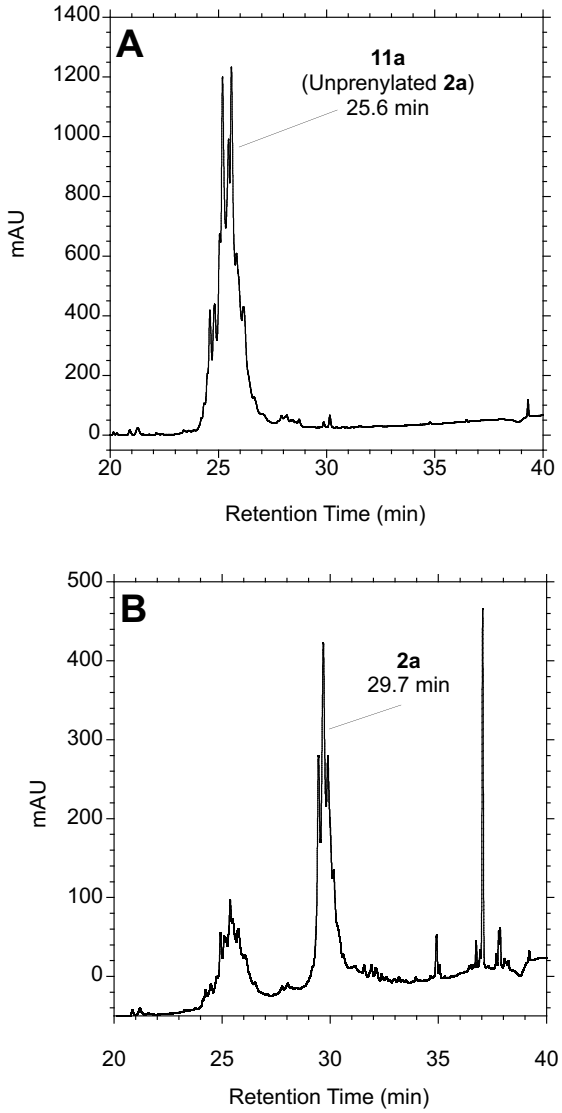

Fig. S5. Prenylation of peptide (**2a**) using $Zn(OAc)_2$ mediated reaction and farnesyl bromide. $Zn(OAc)_2$ coordinates with the thiol at pH 5.0, modulating its nucleophilicity to be able to react with farnesyl bromide and displace the halide. (**A**) Shows LC-MS chromatogram of the precursor to peptide (**2a**) with free thiol. (**B**) shows LC-MS chromatogram of the peptide after prenylation, affording peptide (**2a**). Characteristic retention time shift and MS confirmed the correct product. UV absorbance was monitored at 220 nm. The gradient used was as follows: The gradient used was as follows: 1-10 min, hold at 1% B. 10-35 min, gradient to 100% B. 35-40, hold at 100% B (column wash). 40-41, ramp to 1% B. 41-51, hold at 1% B (column equilibration). The increased hold time at the beginning of the method was essential to remove the DMF fully, enhancing the resolution.

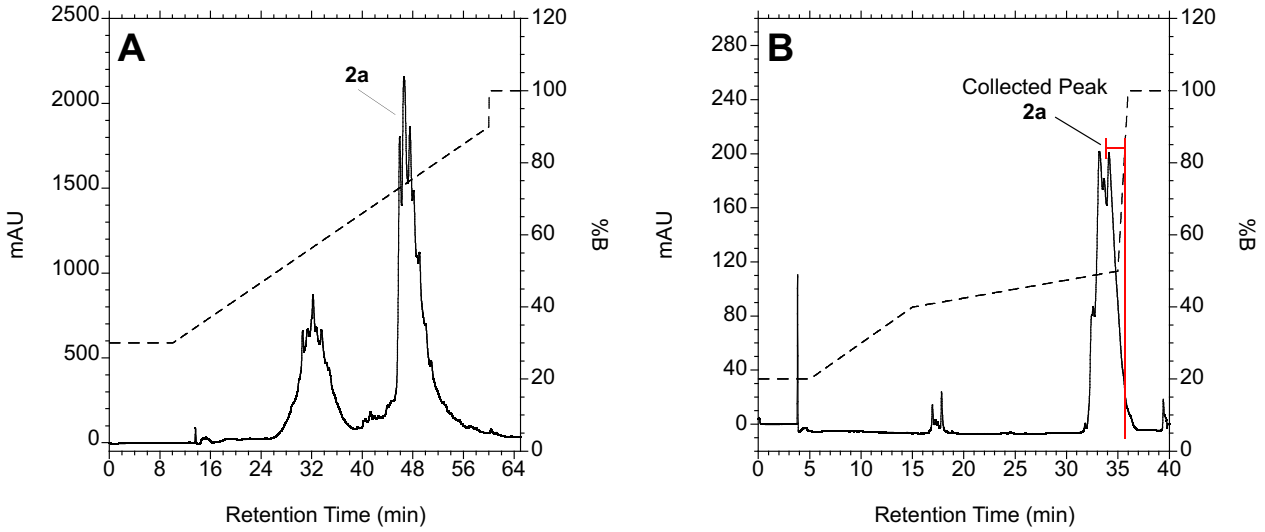

Fig. S6. Two-stage purification of peptide (**2a**). (**A**) Prep purification of peptide (**3a**). This initial stage helped concentrate the desired peptide. 73% purity was obtained afterwards. (**B**) Semi-prep purification using targeted gradient. This stage helped bring the purity to > 95%. This the shallow gradient helped improve the separation between closely eluting truncated peptide materials. UV absorbance was monitored at 280 nm, which helped improve the observed resolution.

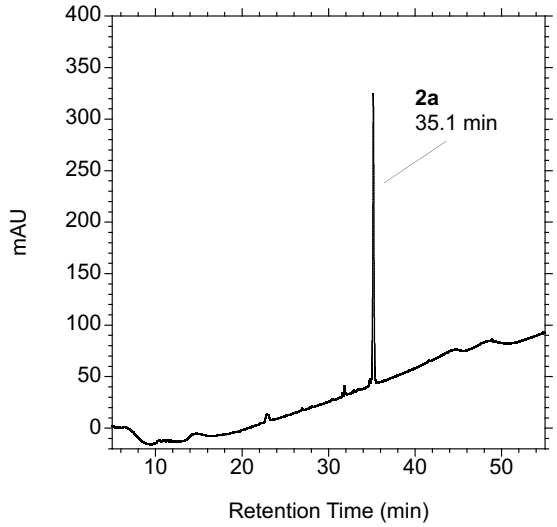

Fig. S7. LC-MS chromatogram of peptide **2a** after two-stage HPLC purification. UV absorbance was monitored at 220 nm. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

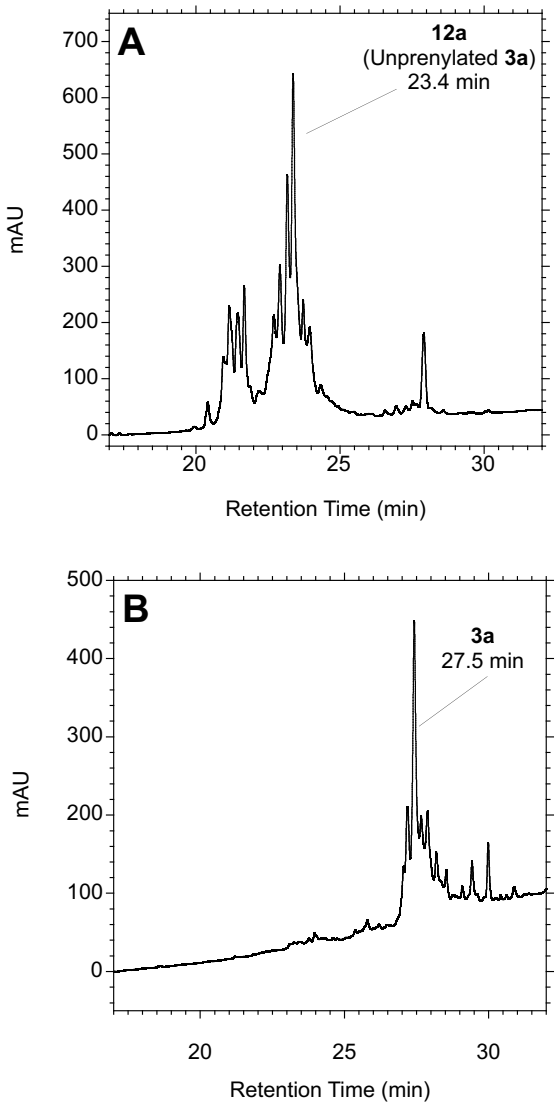

Fig. S8. Prenylation of peptide (**3a**) using $Zn(OAc)_2$ mediated reaction and farnesyl bromide. $Zn(OAc)_2$ coordinates with the thiol at pH 5.0, modulating its nucleophilicity to be able to react with farnesyl bromide and displace the halide. (**A**) Shows LC-MS chromatogram of the precursor to peptide (**3a**) with free thiol. (**B**) shows LC-MS chromatogram of the peptide after prenylation, affording peptide (**3a**). Characteristic retention time shift and MS confirmed the correct product. UV absorbance was monitored at 220 nm. The gradient used was as follows: 1-10 min, hold at 1% B. 10-35 min, gradient to 100% B. 35-40, hold at 100% B (column wash). 40-41, ramp to 1% B. 41-51, hold at 1% B (column equilibration). The increased hold time at the beginning of the method was essential to remove the DMF fully, enhancing the resolution.

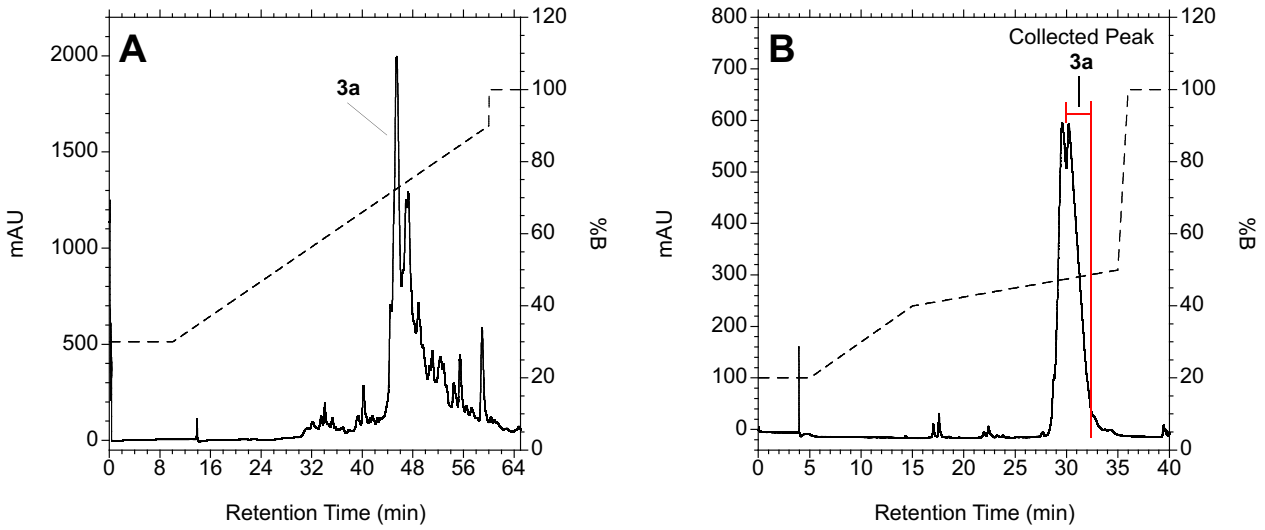

Fig. S9. Two-stage purification of peptide (**3a**). (**A**) Prep purification of peptide (**3a**). This initial stage helped concentrate the desired peptide. 75% purity was obtained afterwards. (**B**) Semi-prep purification using targeted gradient. This stage helped bring the purity to > 95%. This the shallow gradient helped improve the separation between closely eluting truncated peptide materials. UV absorbance was monitored at 280 nm, which helped improve the observed resolution.

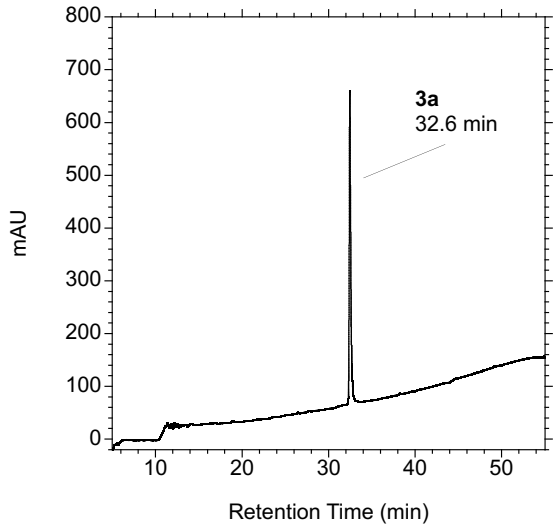

Fig. S10. LC-MS chromatogram of peptide **3a** after two-stage HPLC purification. UV absorbance was monitored at 220 nm. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

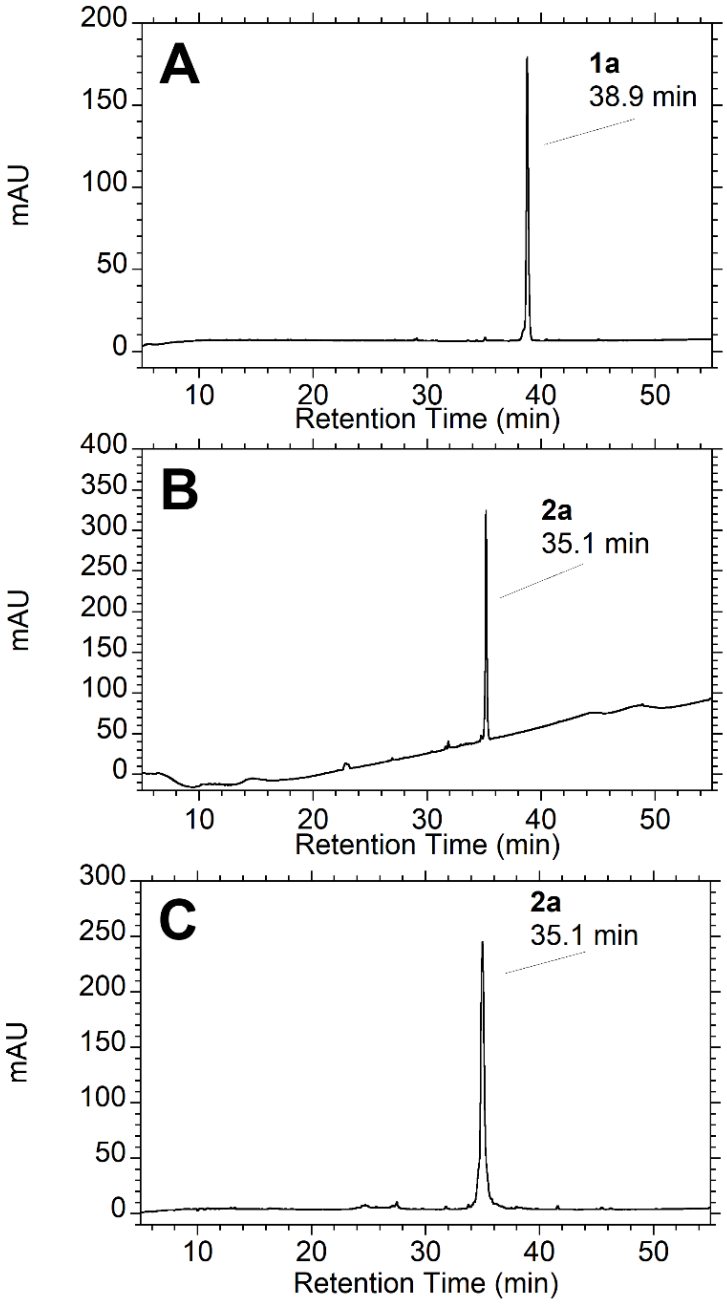

Fig. S11. Saponification reaction of peptide **1a** to obtain **2a**. In light of the significant amount of epimerization observed when producing the C-terminal cysteine containing peptide **2a**, and the minimal amount observed when producing the methyl ester analogue **1a**, a simple saponification reaction with NaOH was used to hydrolyze the methyl ester and convert it to the corresponding carboxylic acid with no epimerization within 1 h. Glacial acetic acid was then used to quench the reaction and prevent any base-mediated epimerization. (**A**) shows the LC-MS chromatogram of peptide **1a** before the reaction. (**B**) shows the LC-MS chromatogram of purified peptide **2a**. (**C**) shows the LC-MS chromatogram of **1a** after the saponification reaction. Both RT and observed mass correspond to the peptide **2a**. UV absorbance was monitored at 220 nm. The gradient used was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

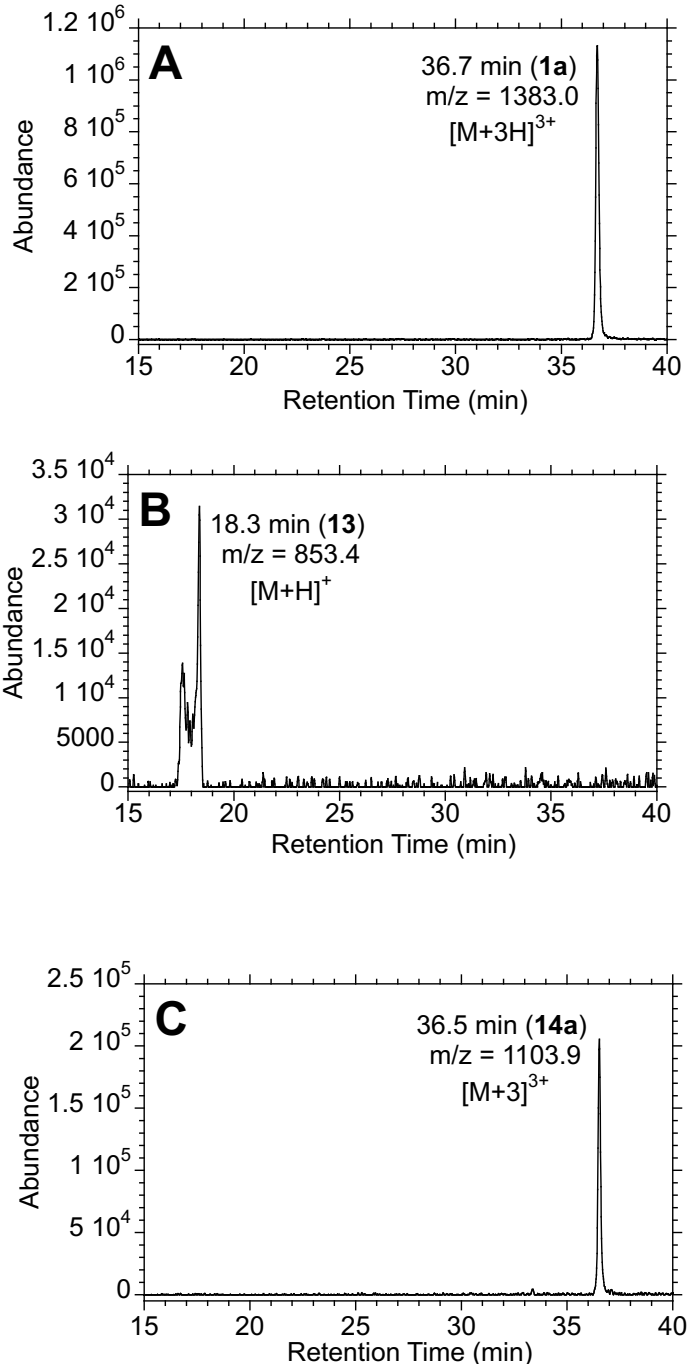

Fig. S12. LC-MS chromatogram of peptide **1a** after reaction with Ste24. Chromatogram shows extracted ions for peptide **3a** (Panel A) as well as the two cleavage products (**13**, Panel B) and (**14a** Panel C) obtained after reacting with Ste24. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

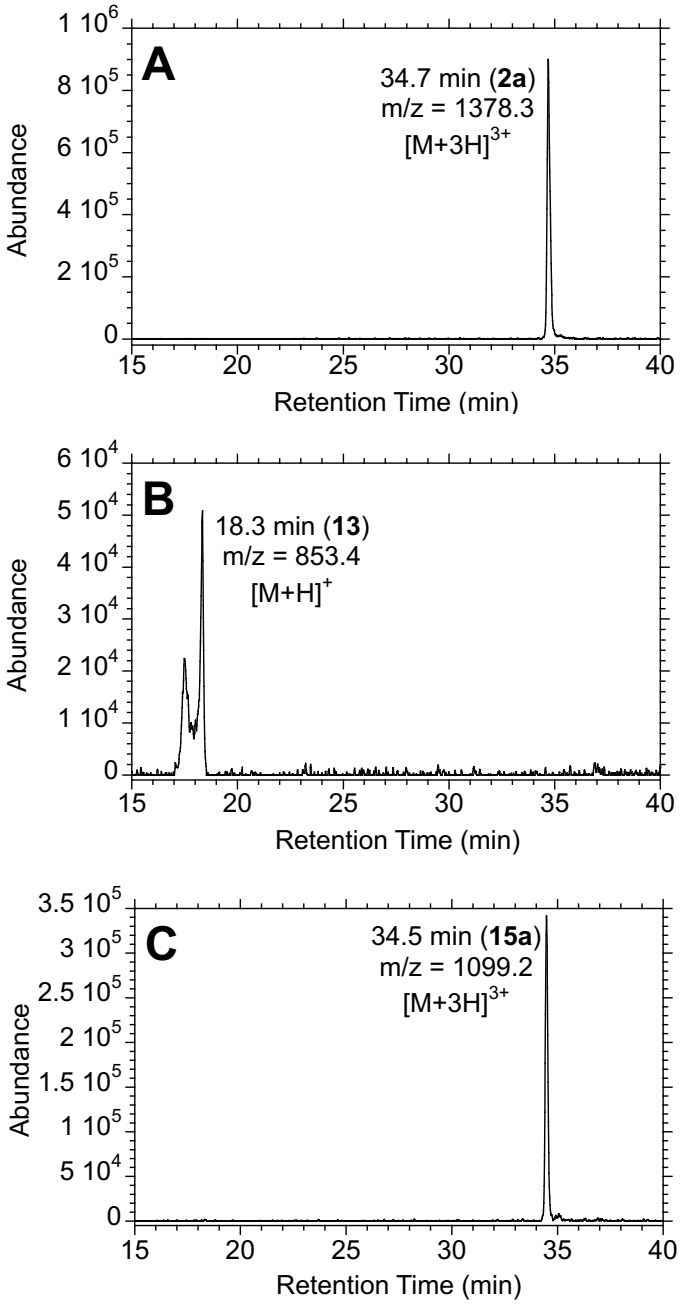

Fig. S13. LC-MS chromatogram of peptide **2a** after reaction with Ste24. Chromatogram shows extracted ions for peptide **2a** (Panel A) as well as the two cleavage products (**13**, Panel B) and (**15a**, Panel C) obtained after reacting with Ste24. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

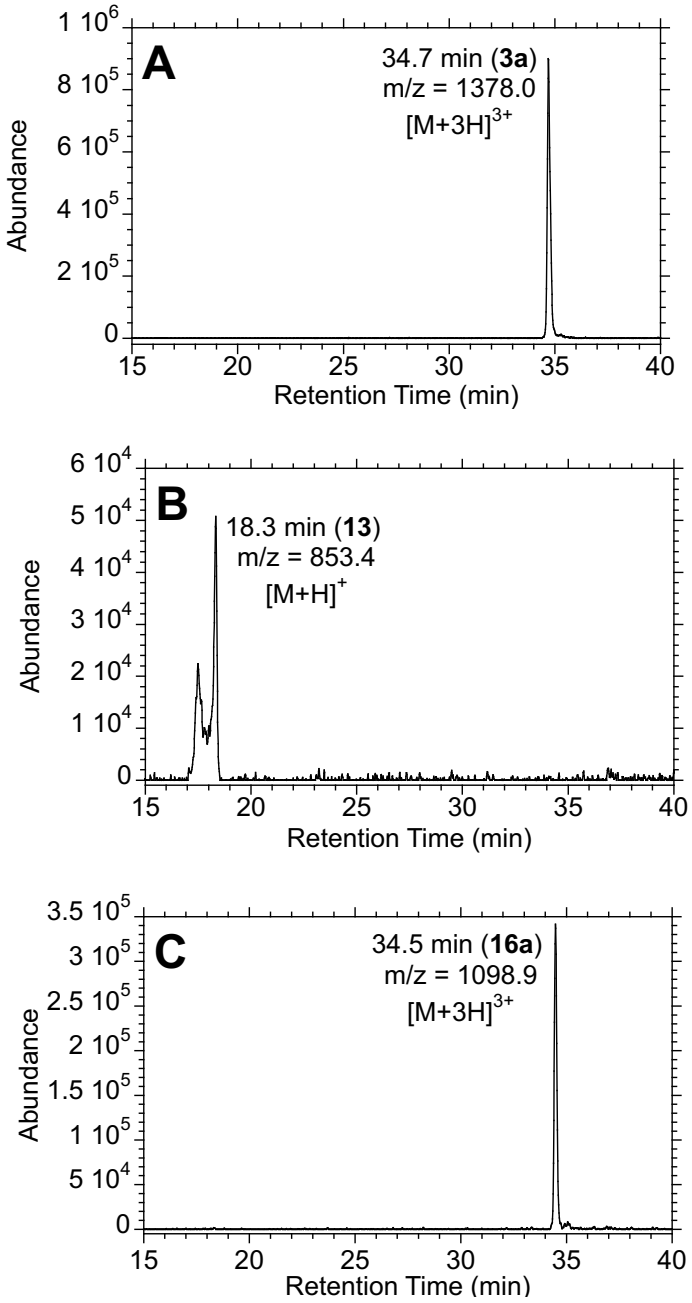

Fig. S14. LC-MS chromatogram of peptide **3a** after reaction with Ste24. Chromatogram shows extracted ions for peptide **3a** (Panel A) as well as the two cleavage products (**13**, Panel B) and (**16a**, Panel C) obtained after reacting with Ste24. The gradient was as follows: 1-5 min, hold at 1% B. 5-55 min, gradient to 100% B. 55-60, hold at 100% B (column wash). 60-61, ramp to 1% B. 61-71, hold at 1% B (column equilibration).

Table S1. Observed MS-MS fragments for peptide **1a** using ETD MS-MS analysis.

Fragment	Charge	Calculated m/z	Found m/z	Fragment	Charge	Calculated m/z	Found m/z
x33	3	1351.6667	1351.6469	c33	3	1275.2871	1275.2817
z32	3	1293.6512	1293.6636	c32	3	1251.6080	1256.6365
z31	3	1251.6369	1251.6285	c31	3	1219.2571	1219.5892
z30	3	1218.6141	1218.9320	c30	3	1180.9148	1180.9123
z29	3	1189.6034	1189.5773	c29	3	1118.8883	1118.8800
z28	3	1155.9209	1155.9145	c28	3	1069.8655	1069.8551
z27	3	1132.2418	1132.2164	c27	3	1036.8427	1036.8409
x26	3	1112.8945	1112.5811	b26	3	1017.1601	1012.4920
z25	2	1611.8167	1611.7800	c25	3	975.1373	975.1588
z24	2	1584.8114	1584.8103	c24	3	937.4426	937.8064
z23	3	1018.8503	1018.8635	c23	3	899.7479	899.4418
z22	3	920.8181	920.4513	c22	3	845.3934	845.3934
z21	3	892.1392	892.4178	c21	3	807.3791	807.3916
z20	3	835.1056	835.1660	c20	2	1144.5383	1144.2338
z19	2	1201.6310	1201.5565	c19	2	1088.5024	1088.5235
z18	2	1158.1150	1158.5679	c18	2	1024.4549	1024.5090
z17	2	1114.5989	1114.5592	c17	3	640.2915	640.2961
z16	2	1050.0776	1050.1871	c16	2	907.9043	907.9564
z15	2	986.0302	986.2214	c15	3	582.2702	582.2886
z14	2	943.4855	943.4625	c14	2	799.8670	799.8987
z13	2	863.9675	863.6716	c13	2	735.8195	735.3139
z12	2	806.9461	806.6434	c12	2	693.8090	693.3177
z11	2	725.4143	725.3608	c11	1	1092.5144	1092.5599
z10	1	1353.7640	1353.7672	c10	1	978.4349	978.4332
z9	1	1223.6534	1223.9624	c9	1	924.4244	924.4443
z8	1	1112.5850	1112.5812	c8	1	836.3608	836.3578
z7	1	1038.5369	1038.4952	c7	1	761.3396	761.3874
z6	2	470.2379	470.2047	c6	2	341.1550	341.2342
z5	2	396.7037	396.1880	c5	1	563.2283	563.2313
z4	1	606.3206	606.2656	c4	2	247.1150	247.1150

z3	1	493.3094	493.2411	c3	2	198.5887	198.1238
z2	1	411.2675	411.2724	c2	1	268.1114	268.1297
z1	1	323.2040	323.2017	c1	1	137.0708	137.0708

Table S2. Observed MS-MS fragments for peptide **2a** using ETD MS-MS analysis.

Fragment	Charge	Calculated m/z	Found m/z	Fragment	Charge	Calculated m/z	Found m/z
z33	3	1332.6595	1332.6552	33			Not Observed
z32	3	1288.9793	1288.9802	c32	3	1251.6080	1251.6304
z31	3	1246.9650	1246.6320	c31	3	1219.2571	1219.6144
z30	2	1820.4097	1820.3731	c30	3	1180.9148	1180.9124
z29	3	1184.9315	1184.9051	c29	3	1118.8883	1118.8619
z28	3	1151.2490	1151.5435	c28	3	1069.8655	1069.5129
z27	2	1690.8512	1690.3446	b27	3	1031.1672	1031.1693
y26	3	1099.5629	1099.5432	b26	2	1517.7365	1517.7223
z25	3	1070.2083	1070.5002	c25	3	975.1373	975.1600
z24	2	1569.2903	1569.2776	c24	3	937.4426	937.4934
z23	3	1014.1784	1014.5152	c23	3	899.7479	899.3314
z22	3	916.1462	916.1950	c22	3	839.7179	839.9102
z21	3	887.4673	887.4362	c21	3	807.3791	807.4041
z20	2	1245.1469	1245.6198	b20	2	1144.5383	1144.5688
z19			Not Observed	c19	2	1088.5024	1088.5303
z18	2	1151.1071	1151.2599	c18	2	1024.4549	1024.5078
z17	2	1107.5911	1107.5679	c17	2	959.9336	959.5426
z16	2	1043.0698	1043.1793	c16	2	916.4176	916.4473
y15	2	987.5356	987.5142	c15	2	872.9017	872.7761
z14	3	609.9845	609.3318	a14	1	1598.7268	1598.7678
y13	1	1729.9386	1729.9228	a13	1	1470.6319	1470.7355
z12	1	1598.8691	1598.7678	c12			Not Observed
z11	2	718.4065	718.3020	c11	1	1092.5144	1092.5540

z10	3	441.5788	441.2156	b10	1	978.4350	978.4345
z9	1	1209.6377	1209.5897	b9	1	907.3979	907.3953
z8	1	1081.5428	1081.5272	c8	1	853.3874	853.4198
z7	1	1024.5212	1024.5081	c7	1	761.3396	761.3654
y6	1	942.4794	942.4792	b6	1	664.2760	664.2719
y5	1	795.4110	795.4091	b5	1	563.2283	564.2758
z4	1	592.3049	592.2218	c4	1	493.2227	493.2413
y3	1	494.3048	494.3051	c3	1	396.1700	396.1877
z2	1	380.2253	380.1505	c2	1	268.1114	268.1289
y1	1	326.2149	326.2131	c1	1	137.0708	137.1320

Table S3. Observed MS-MS fragments for peptide **3a** using ETD MS-MS analysis.

Fragment	Charge	Calculated m/z	Found m/z	Fragment	Charge	Calculated m/z	Found m/z
z33	3	1332.3315	1332.1702	b33	3	1269.6115	1269.6300
z32	3	1288.6513	1288.6543	c32	3	1251.6080	1251.6386
z31	3	1246.6370	1246.6324	c31	3	1219.2571	1219.3054
z30	3	1213.6142	1213.6151	c30	3	1180.9148	1180.9154
z29	3	1184.6035	1184.5802	c29	3	1118.8883	1118.8682
z28	3	1150.9210	1150.8749	c28	2	1604.2946	1604.3367
z27	2	1690.3592	1690.3324	b27	3	1031.1672	1031.1912
y26	3	1099.2349	1099.5608	c26	3	1017.8356	1017.5219
z25	2	1604.3168	1604.3367	c25	3	975.1373	975.1573
z24	2	1568.7983	1568.7910	c24	2	1397.1469	1397.6271
z22	3	915.8182	915.7011	c23	3	899.7479	899.4703
z21	3	887.1393	887.4221	b22	3	839.7179	839.9160
x20	3	844.4410	846.4503	c21	3	807.3791	807.4004
x19	3	810.7584	810.9002	b20	2	1144.5383	1144.5738
z18	2	1150.6151	1150.5683	c19	2	1088.5024	1088.5258
z17	2	1107.0991	1107.0703	c18	2	1024.4549	1024.5184
z16	2	1042.5778	1042.8577	c17	3	640.2915	646.2666
z15	2	978.5303	978.4321	c16	2	916.4176	916.4459

x14	2	935.9857	935.8061	c14	2	822.3778	1822.3585
z13	2	856.4677	856.6807	a13	2	735.8196	735.3171
z12	1	1597.8851	1597.7700	12			Not Observed
y11	1	1451.8484	1451.7880	b11	1	1075.4878	1075.5293
z10	1	1321.7378	1321.6433	b10	1	978.4350	978.4321
z9	1	1208.6537	1208.5967	c9	1	924.4244	924.4667
z8	1	1080.5588	1081.5489	c8	1	853.3874	853.6814
z7	1	1023.5372	1023.5142	b7	1	735.3131	735.3171
z6	1	924.4688	941.4904	b6	1	664.2760	664.2754
z5	1	777.4004	777.3747	b5	1	563.2283	563.3242
z4	1	591.3209	591.3246	c4	1	493.2227	493.3217
z3	1	478.3097	478.1025	c3	1	396.1700	396.1892
z2	1	379.2413	379.1461	c2	1	268.1114	268.1291
z1	1	322.2200	322.1765	c1	1	137.0708	137.1320