Supporting Information

Detection of Alkynes via Click Chemistry with A Brominated Coumarin Azide by Simultaneous Fluorescence and Isotopic Signatures in Mass Spectrometry

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1. Analytical Methods

1.1 LC/ELSD/DAD/MS Analysis

Brominated coumarin azide (3-azido-6-bromo-7-hydroxy-chromen-2-one, CAS number 1352503-77-5, vendor Princeton BioMolecular Research, Inc., catalog number W8595, Princeton, NJ) was analyzed by a Waters Acquity Classic UPLC with a reverse-phase column (Waters Cortecs, C18, 2.1 x 30 mm) and detected by evaporative light scattering (ELSD), diode array (DAD) at 210-400 nm, and Mass Spectrometry (Waters Acquity SQD2) with positive/negative electrospray ionization scanning between 100-1300 Da. The UPLC used 10 mM ammonium acetate in water as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1 mL/min. The gradient was initiated at 5% of mobile phase A followed by a linear increase to 60% of mobile phase B in 1.6 minutes, then to 95% mobile phase B by 2.2 minutes, and returned to 5% mobile phase B over 1 minute.

1.2 UV/Vis Absorption Spectra of Brominated Coumarin Triazole Click Product

HPLC-DAD Chromatography

The triazole click product was analyzed on an Agilent 1100 LC-DAD using a reverse-phase column (ThermoFisher Scientific, MAbPac™ RP LC, 2.1x100 mm). The chromatography was conducted at a flow rate of 1.0 mL/min. The gradient program was initiated with 2% mobile phase B (0.08% formic acid plus 0.02% trifluoroacetic acid in acetonitrile) and held for 5 min, followed by a linear increase to 40% mobile phase B over 25 minutes, then to 65% mobile phase B over 10 minutes, further to 98% mobile phase B, held for 10 minutes, and finally returned to 2% mobile phase B with a 10-minute hold. The triazole click product was separated from the matrix components and its UV/vis intensity-based absorption spectrum was generated using Agilent software.

Determination of Extinction Coefficient of Brominated Coumarin Triazole

The acylated model peptide alkyne contained a tryptophan chromophore that absorbed UV at 280 nm with known extinction coefficient (Ex. Co.) of 5500 $M^{-1}cm^{-1}$.[1] To determine the contribution of the brominated coumarin triazole to 280 nm absorption, first, the intensity-based absorption spectrum of the brominated coumarin triazole from propargyl alcohol, which does not contain a chromophore that absorbs at either 280 nm or 350 nm, was generated, and the ratio of absorbance at 280 nm to 350 nm was calculated. Second, the intensity-based absorbance spectrum of brominated coumarin triazole tryptophan from the model peptide alkyne was generated. In addition to absorption at 350 nm, this spectrum contained absorption at 280 nm contributed by both the tryptophan in the model peptide and the chromophore in the brominated coumarin triazole. The difference in absorption at 280 nm between the two triazole click products is contributed by the tryptophan. The concentration of the model peptide alkyne was deduced using the Beer Lambert Law.[2] The extinction coefficient of the brominated coumarin triazole absorption at 350 nm was then calculated to be 18,522 M^{-1} cm^{-1}. Finally, the UV/vis extinction coefficient-based spectrum for brominated coumarin triazole was generated.

$A = \varepsilon * b * c$

A: absorbance (difference between the two click products);

Ɛ=Ex. Co. (5500 M-1 cm-1 for the model peptide);

b: path length (1 cm);

c: concentration of the model peptide alkyne (calculated).

1.3 Scanning of Excitation and Emission Wavelength

The fluorescence properties of brominated coumarin triazole click products generated from five alkynes were examined in the following three solutions: LC mobile phase A (0.085% formic acid and 0.02% trifluoroacetic acid in water, pH ~2), 85% mobile phase A and 15% mobile phase B (0.08% formic acid plus 0.02% trifluoroacetic acid in acetonitrile, pH ~3), and 75% mobile phase A and 30% mobile phase B (pH ~4). These solutions represent the product-eluting conditions for LC-fluorescence-MS analysis, in which the buffer gradient went from 2% to 30% mobile phase B. The click products were generated in a stock concentration of 500 µM in HEPES buffer and diluted 10-fold with each of the above solutions. Excitation scans were from 250 nm to 600 nm in increments of 1 nm using an emission of 470 nm. Emission scans were from 420 nm to 600 nm at an excitation of 350 nm for pH 2-4.

1.4 Determination of Brominated Coumarin Triazole Click Product Loading on LC

The brominated coumarin triazole click product from the model pepitde alkyne was used to deterimne the limit of detection (LOD) for MS and flourescence. The click product loading quantity on LC was measured using the following equation:[3]

Load (µmole) = flow rate(mL/60s)*peak area/(path length (cm)*ex. co(AU*M-1*cm-1)

Concentration (µM) = load (µmole)/injection volume (µL)*10^6

The initial traizole click product sample was diluted 8-fold and analyzed by LC-UV at 350 nm. The LC parameters are listed below:

Flow rate = 0.05 mL/60s;

Peak area = 599905µAU*s

Ex Co = 18522 AU*M-1*cm-1

Path length = 1 cm

Injection volume = 10 µL

The column loading was calculated to be 27.0 pmole, and the concentration of the intial triazole click product sample was 21.6 µM.

2. Characterization of Brominated Coumarin Azide

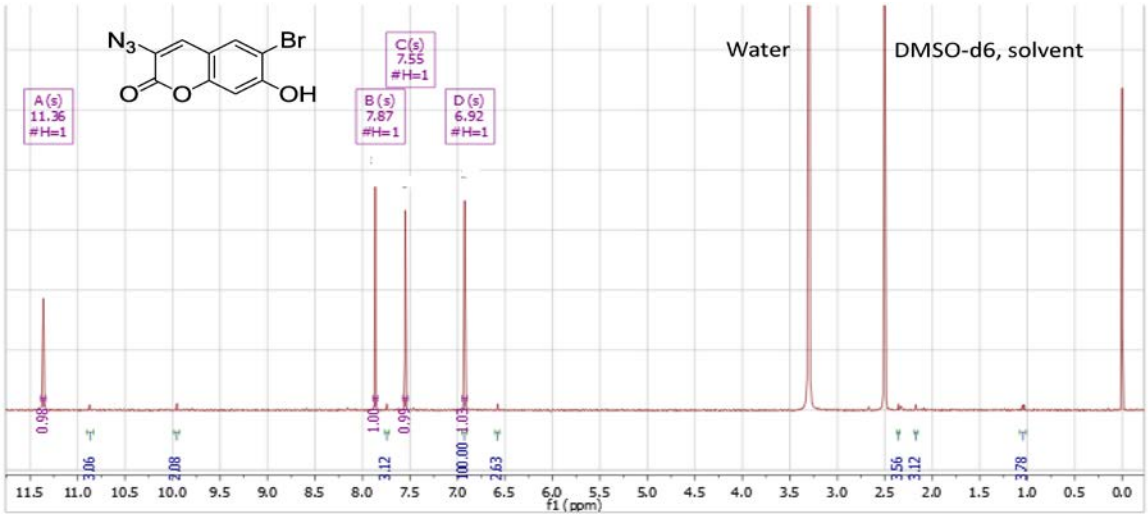


Figure S1. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.36 (s, 1H), 7.87 (s, 1H), 7.55 (s, 1H), 6.92 (s, 1H). The purity was determined to be greater than 95%.

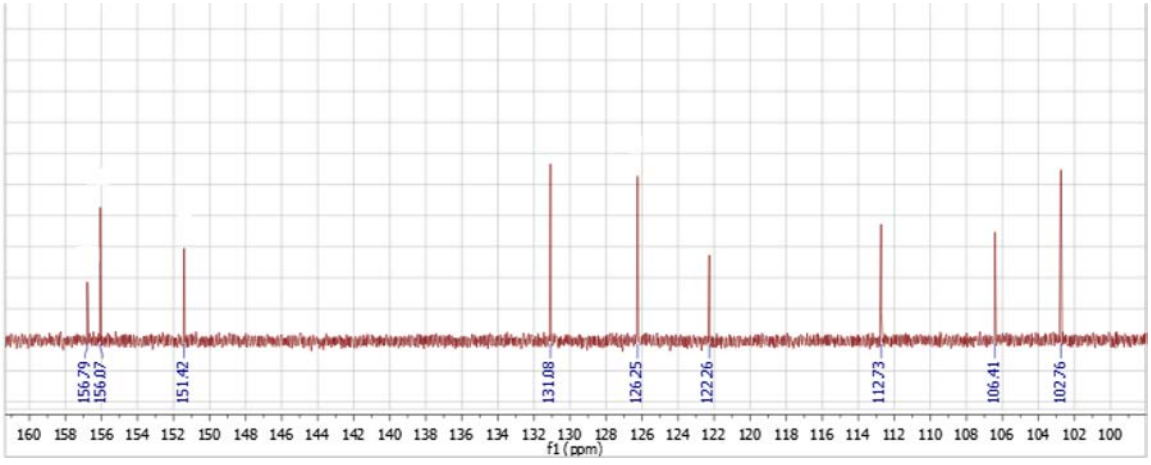


Figure S2. ^{13}C NMR (101 MHz, DMSO-d6) δ 102.76, 106.41, 112.73, 122.26, 126.25, 131.08, 151.42, 156.07, 156.79

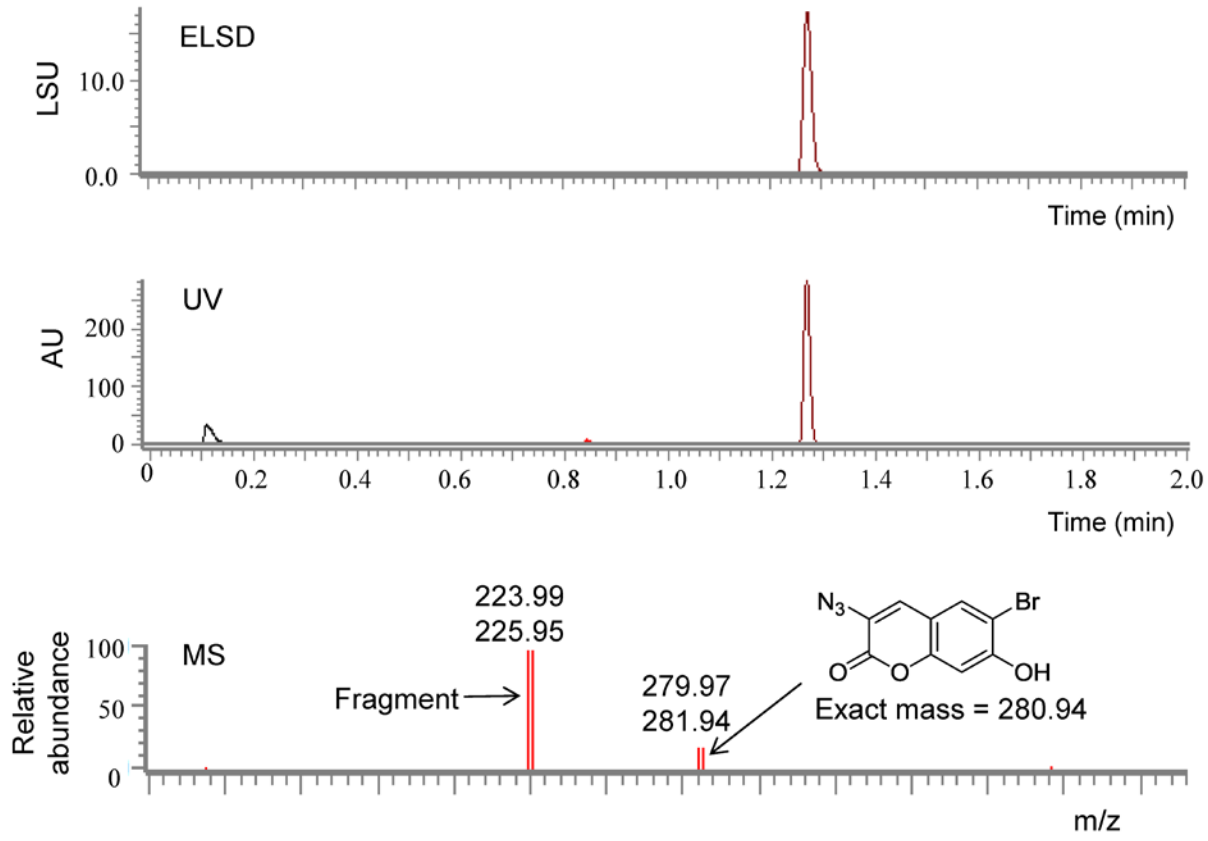


Figure S3. Purity of the Brominated Coumarin Azide. The purity of the brominated coumarin azide was 100% and 96% as detected by ELSD (top) and DAD (middle), respectively. The observed pair of ions with m/z = 279.97 and 281.94 is consistent with the $[M-H]^-$ molecular ion and the doublet isotopic pattern of the brominated product with an exact mass of 280.94 (bottom).

3. Characterization of Brominated Coumarin Triazole Click Product

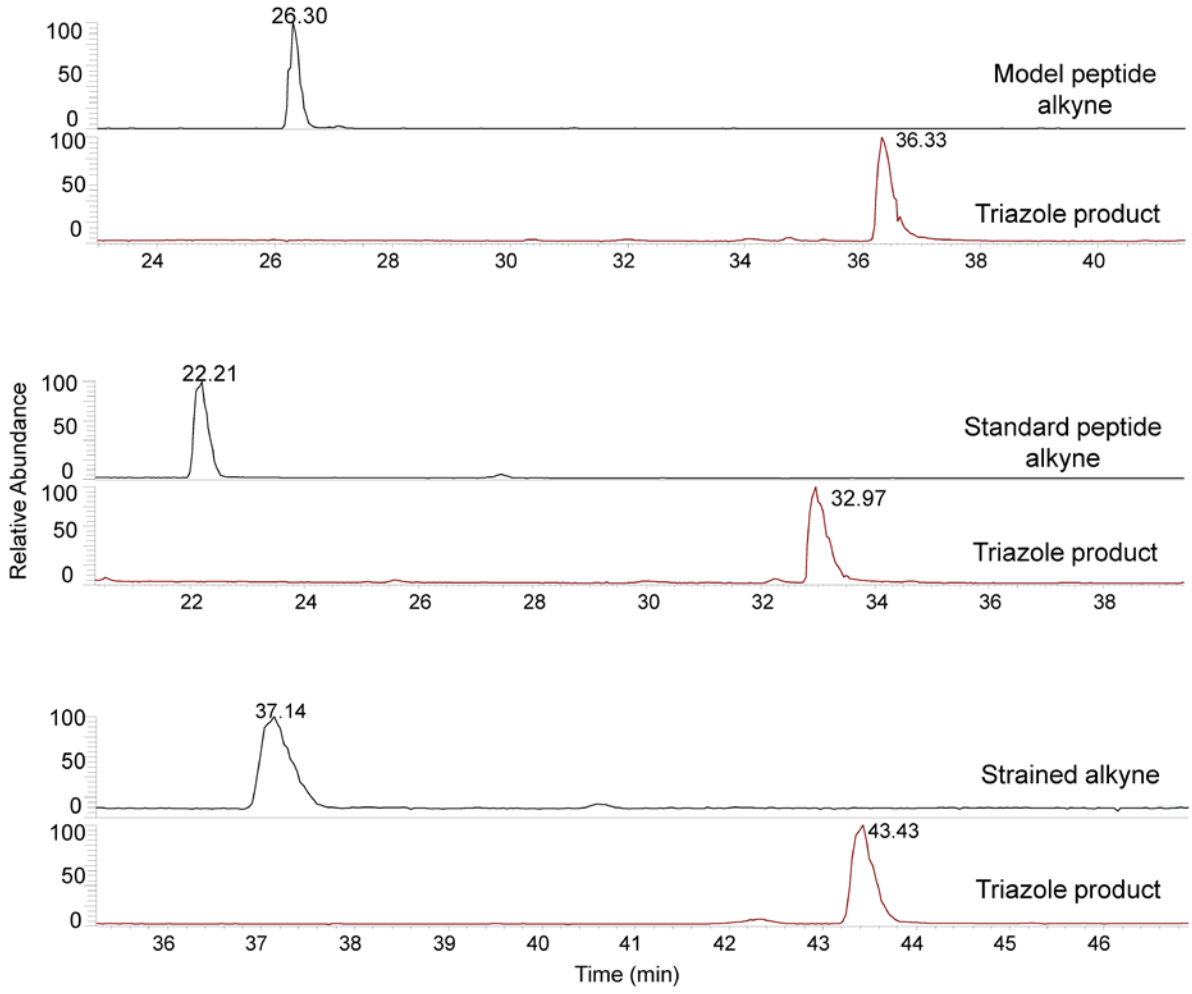


Figure S4. Click Reaction Yield. MS Total Ionization Chromatogram (TIC) of the peptide alkyne (black) and its triazole click product (red). The pair is shown for the model peptide alkyne (top), a standard peptide alkyne (middle) and the strained alkyne (bottom). There was no peptide alkyne detected in the triazole click product for any pair, suggesting the reaction was nearly 100%.

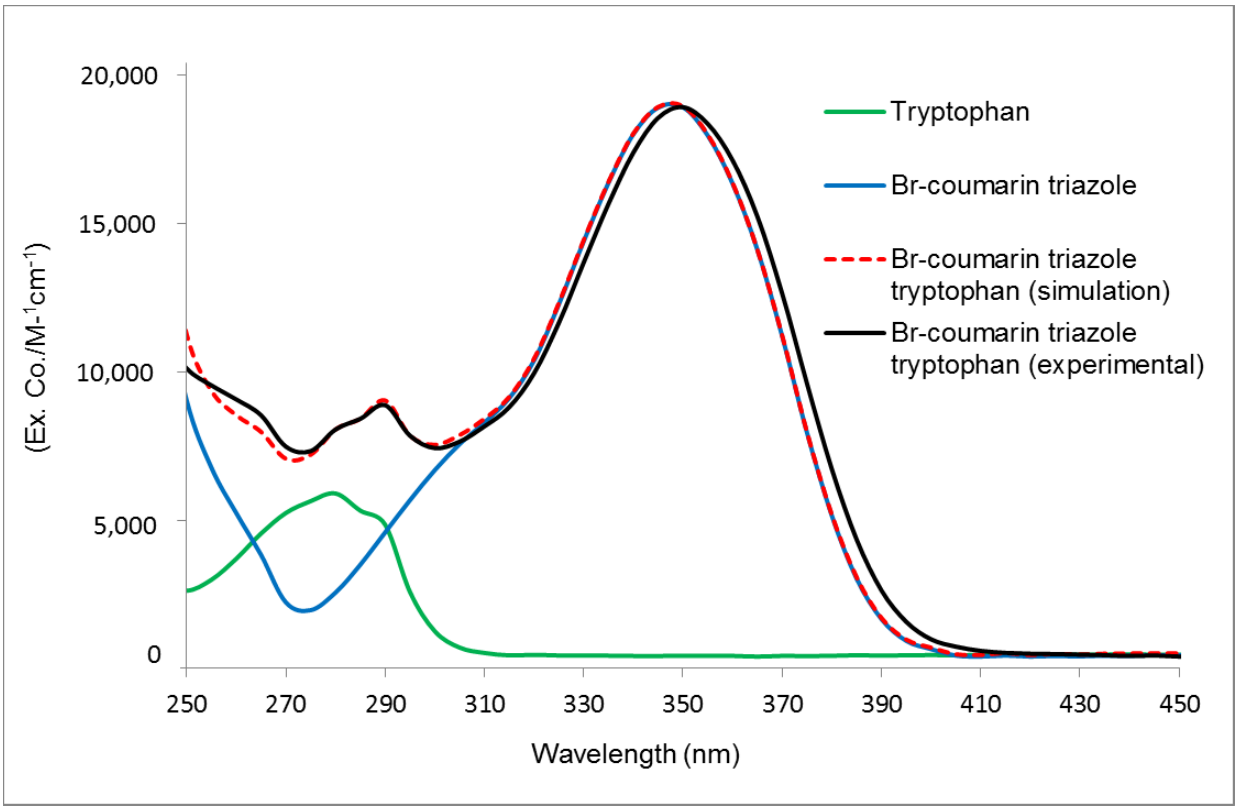


Figure S5. UV/vis Ex. Co. Based Absorption Spectra of Click Reaction Materials. Tryptophan in the model peptide absorbs at 280 nm (green) and the brominated coumarin triazole has a maximum absorbance at 350 nm (blue) and absorbs at 280 nm. The brominated coumarin triazole tryptphan absorbs at 280 nm, contributed by the tryptophan and the brominated coumarin triazole via mathematical addition (red dotted). The brominated coumarin triazole tryptophan spectrum from experimental data is shown in black. The simulation and experimental data matched very well, suggesting that the calculated Ex. Co. of brominated coumarin triazole was accurate.

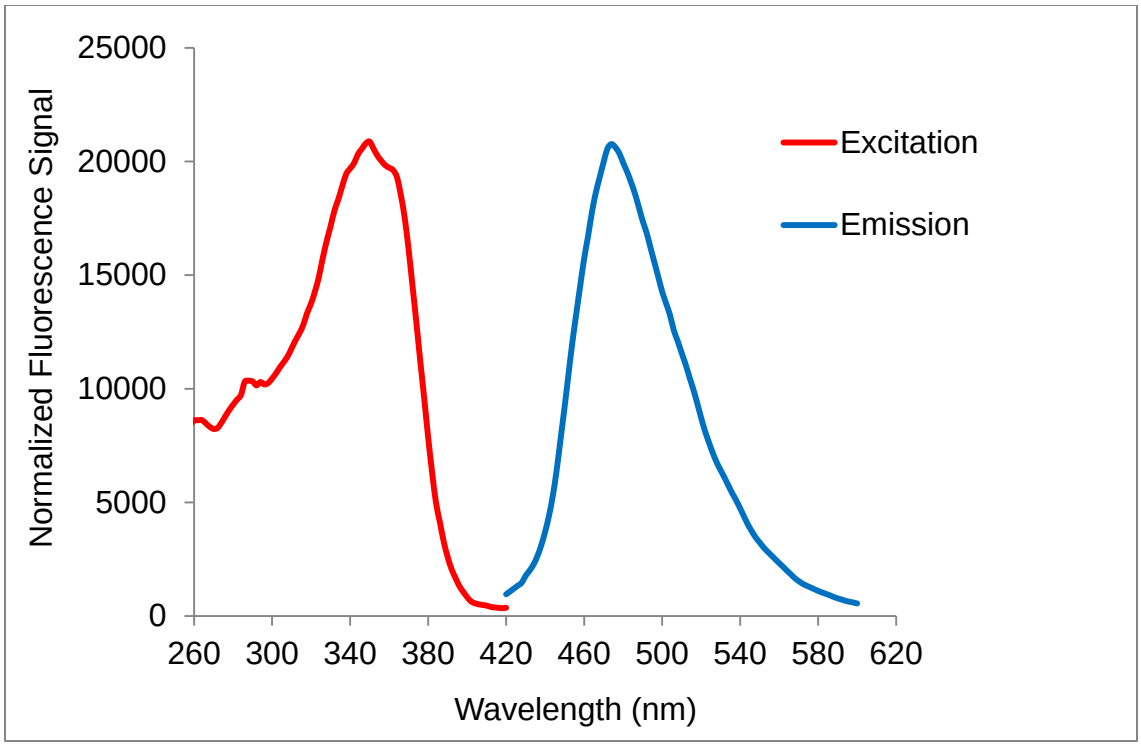


Figure S6. Excitation and Emission Wavelengths of Brominated Coumarin Triazole Products from the Model Peptide Alkyne. The maximum excitation and emission wavelengths are 350 nm and 470 nm, respectively, at pH 2-4, with data at pH 4 shown in the figure. The same results were observed for products from the other four alkynes with the exception that the maximum emission wavelength for the strained alkyne was 460 nm (data not shown). The pH for LC-MS acquisition had no effect on the absorbance spectrum of the brominated coumarin triazole, and the functional group had minimal effect.

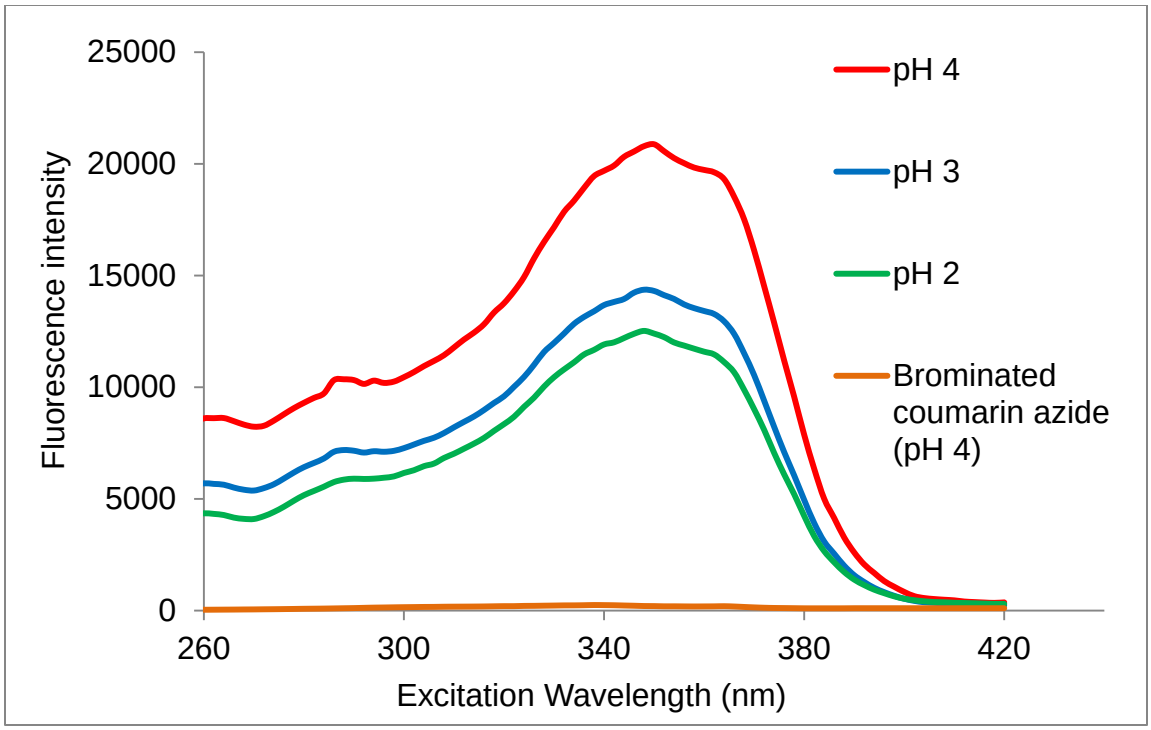


Figure S7. Effect of pH on Fluorescence Intensity. Excitation spectra of the triazole product from the model peptide alkyne at pH 2, 3 and 4. The brominated coumarin azide itself is not fluorescent.

Native peptide Brominated peptide

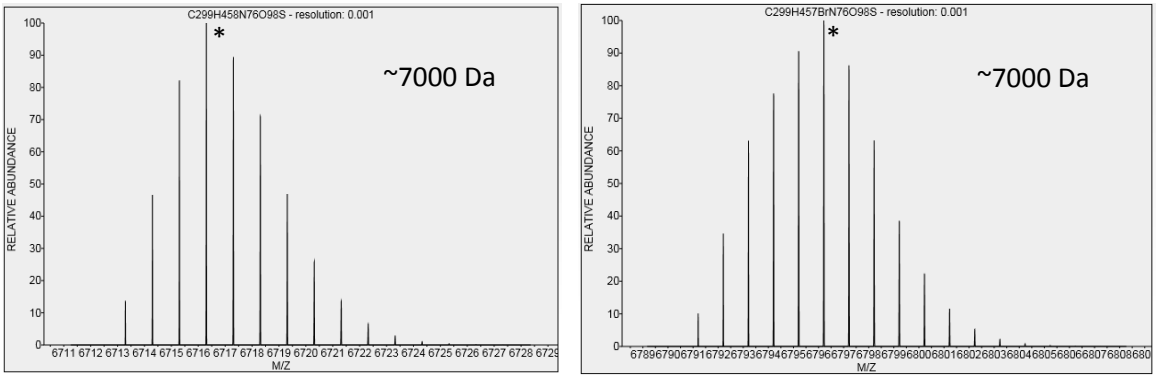


Figure S8. Isotopic Pattern of Brominated Coumarin Triazole Products Across a Wide Molecular Weight Range. The MS precursor ion spectra up to ~7000 Da were from simulated results using ChemCalc (Institute of chemical sciences and engineering).

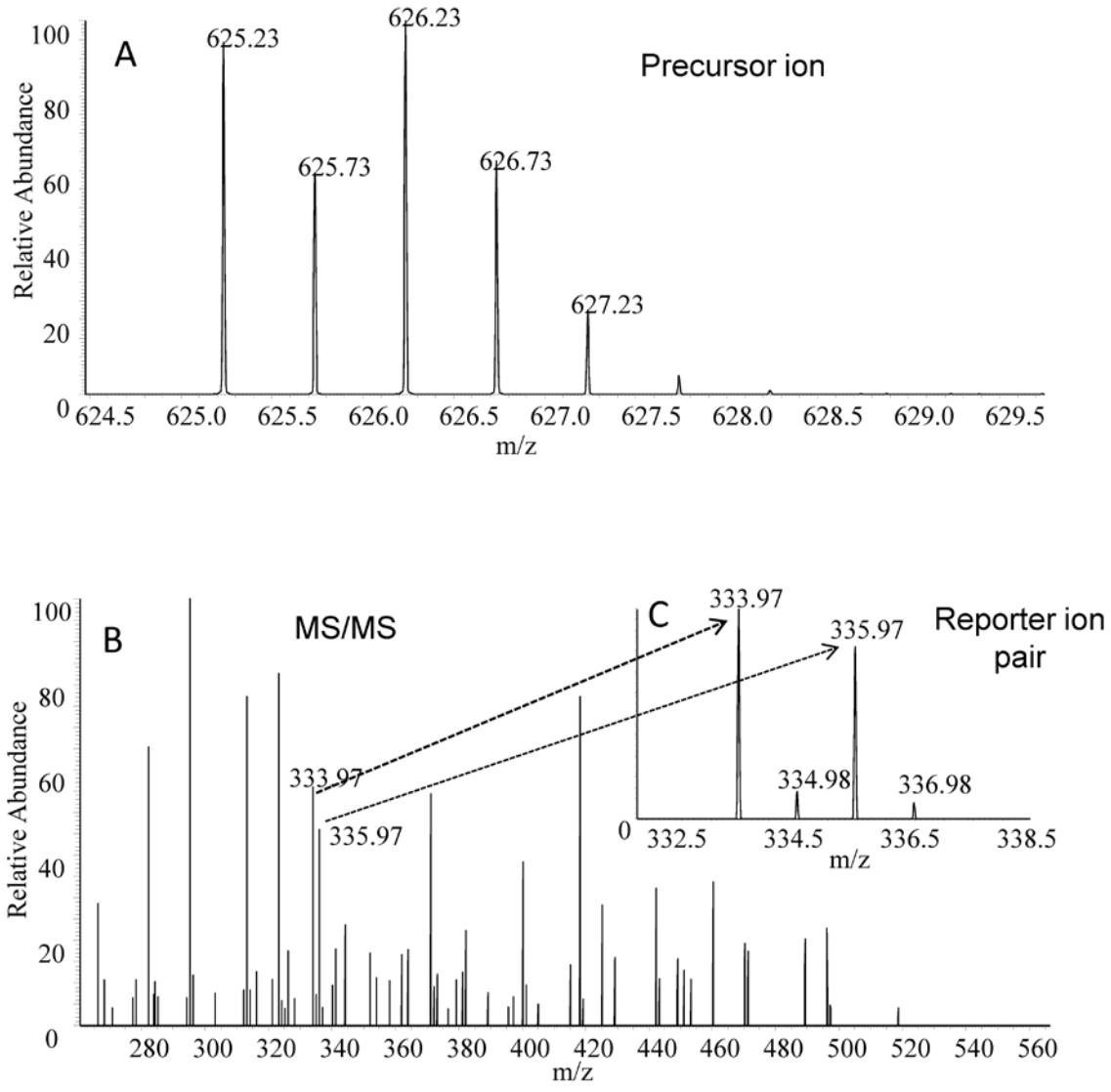
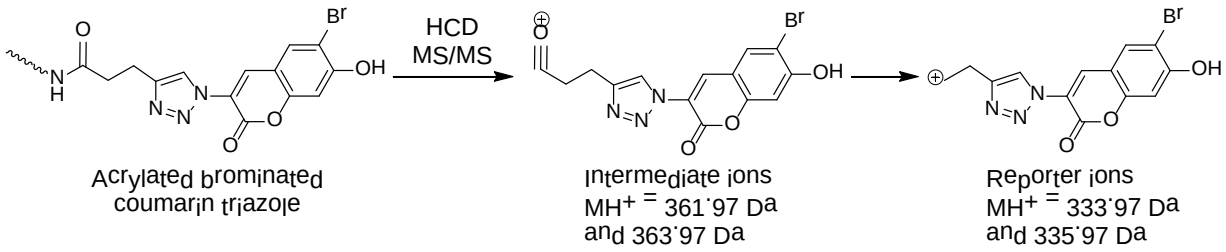


Figure S9. Reporter Ions of m/z = 333.97 and 335.97 from the Acylated Lysine Model Brominated Coumarin Triazole. Precursor ion spectrum (A), coresonding MS/MS spectrum (B) and reporter ion pair (C). The reporter ion pair is more intense in a HCD than CID spectrum.



Scheme S1. Formation of the Reporter Ion Pair during Fragmentation of the Acylated Triazole. The acylated triazole was cleaved from the peptide at the amide bond and then futher lost a carbonyl group to generate the reporter ion pair.

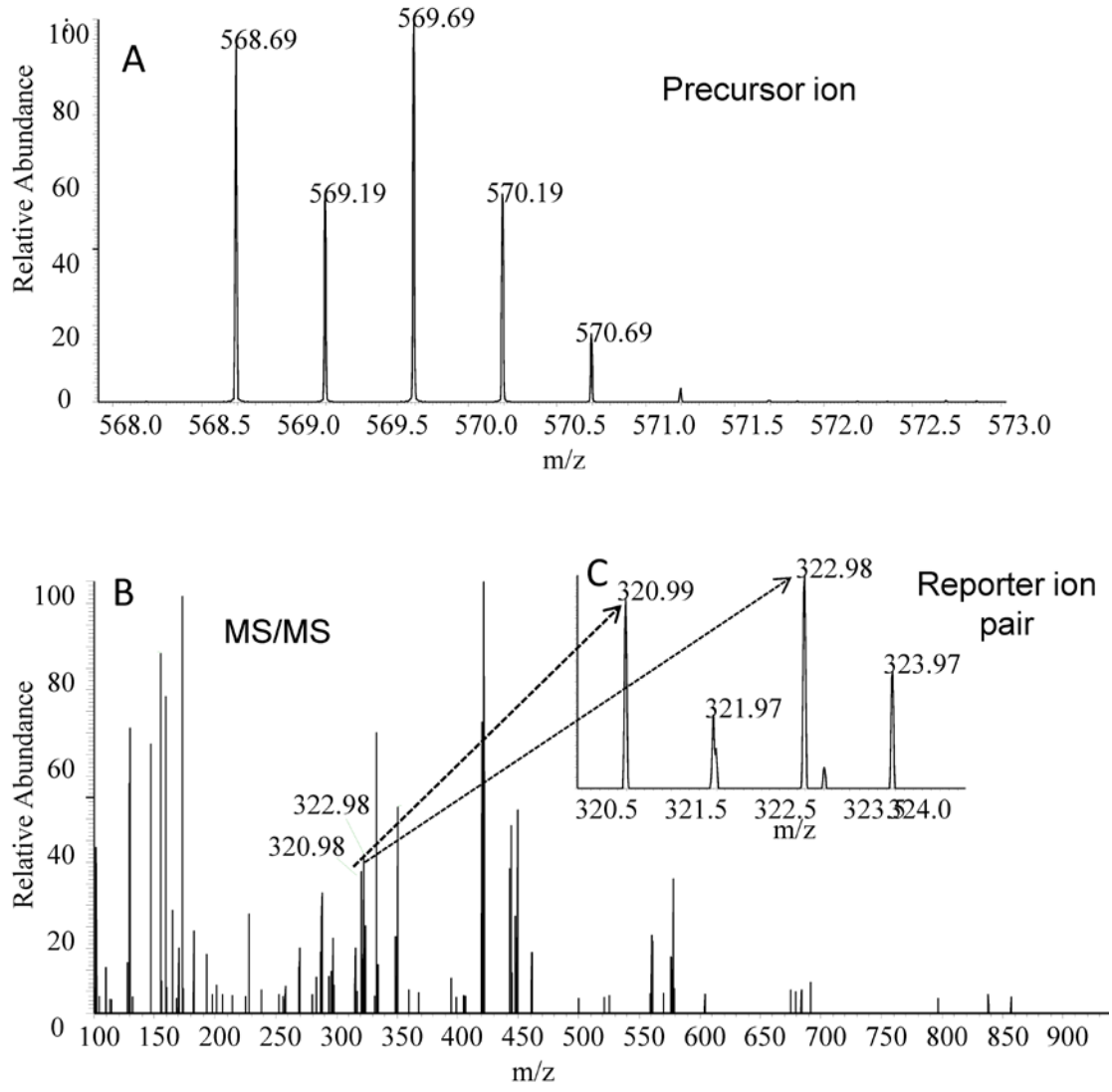
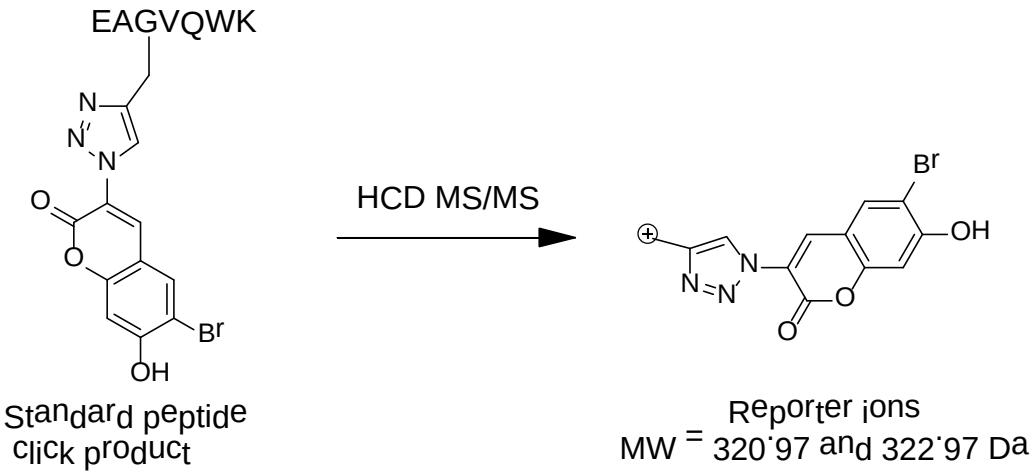


Figure S10. Reporter Ions of m/z = 320.99 and 322.98 from a Standard Alkyne-Brominated Coumarin Triazole Click Product. Precursor ion spectrum (A), corresonding MS/MS spectrum (B) and reporter ion pair (C).



Scheme S2. Formation of the Reporter Ion Pair during Fragmentation of the Brominated Coumarin Triazole from the Standard Peptide Alkyne. The carbon bond adjacent to the triazole ring was cleaved to form the reporter ion pair of 320.97 and 322.97, which resulted from the bromine isotopic signature.

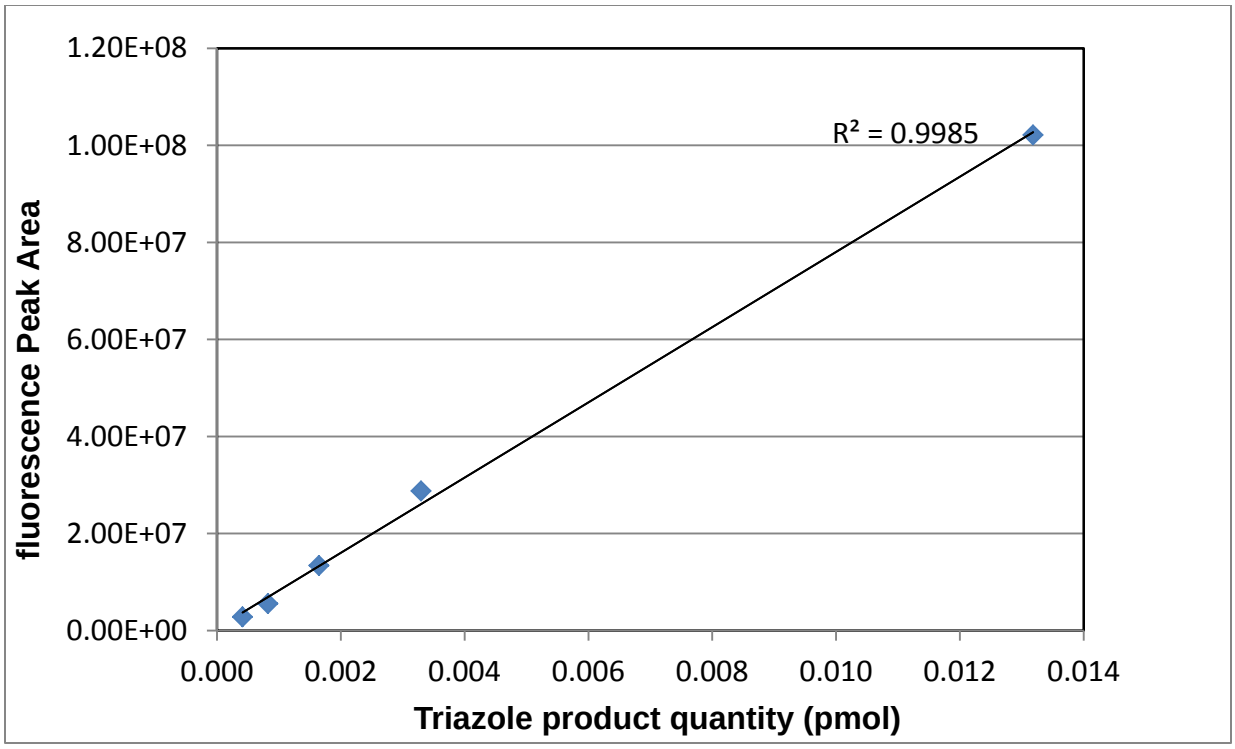


Figure S11. Fluorescence LOD of the Brominated Coumarin Triazole. The fluorescence peak area versus the quantity of brominated coumarin triazole click product from the model peptide. The curve was constructed using the 5 lowest points. The lowest quantity of 0.41 fmol with S/N ratio of 8 was defined as the LOD because the point below that quantity was not detectable. The product quantity was determined using the equations in the column loading section (section 1.4).

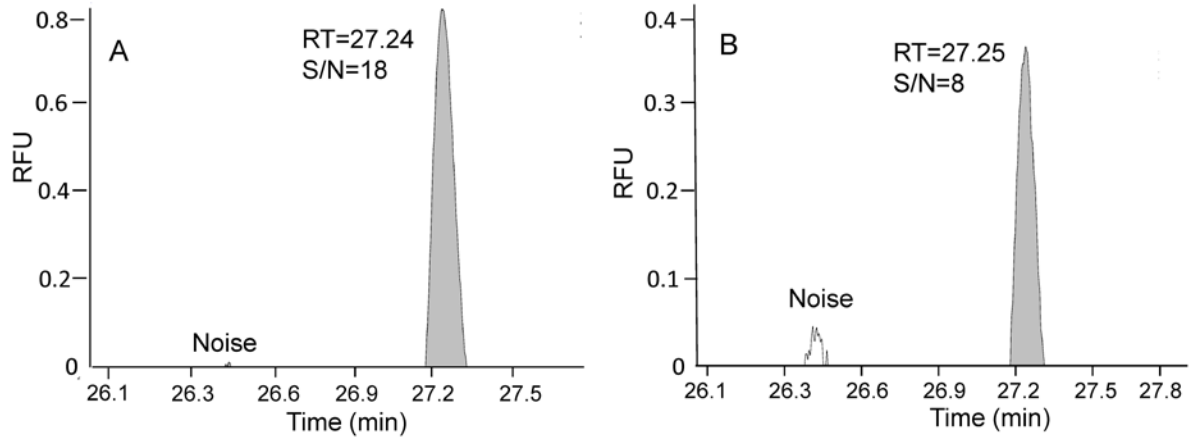


Figure S12. Fluorescence LOD Peaks of the Brominated Coumarin Triazole. The penultimate lowest fluorescence peak (A) and the lowest fluorescence peak (B) used for LOD determination.

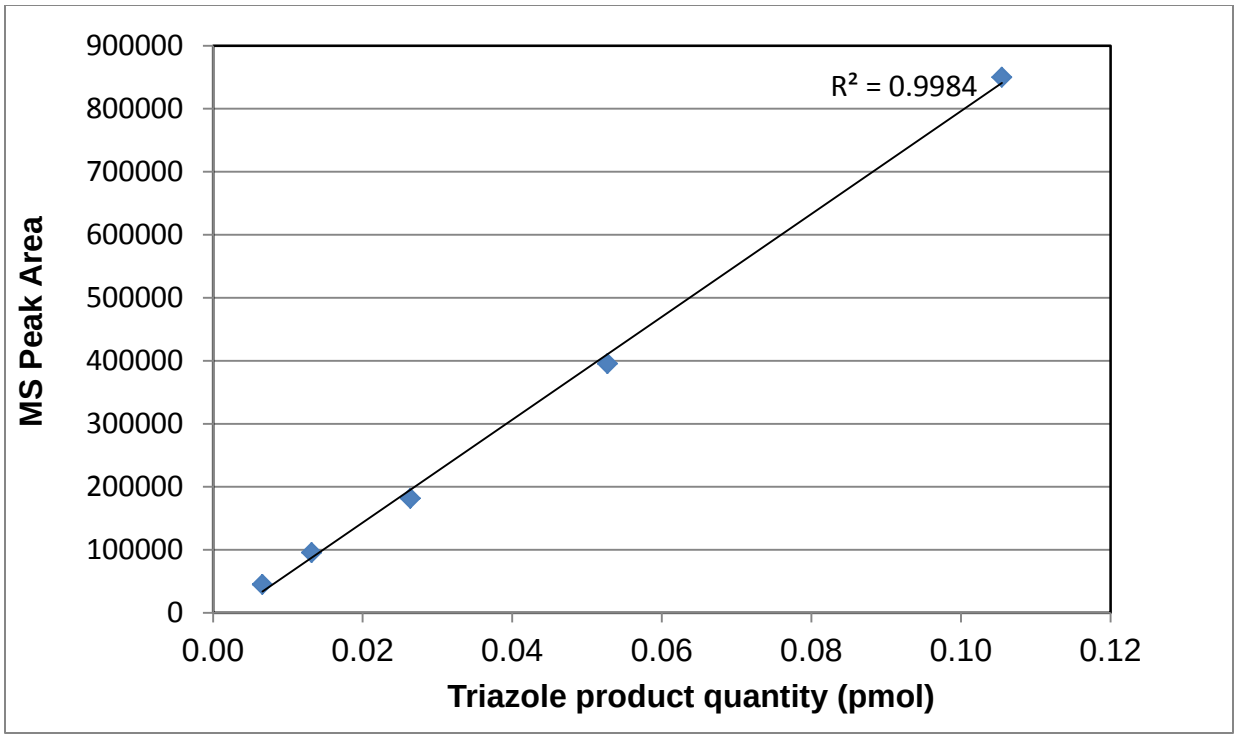


Figure S13. MS Limit of Detection (LOD) of the Brominated Coumarin Triazole. The MS peak area versus the quantity of brominated coumarin triazole click product from the model peptide. The sum of the peak area from the extracted ion chromatogram (XIC) was obtained from m/z 625.1 to 627.3. The curve was constructed using the 5 lowest points. The lowest quantity of 6.6 fmol with S/N ratio of 8 was defined as the LOD because the point below that quantity was not detectable. The product quantity was determined using the equations in the column loading section (section 1.4).

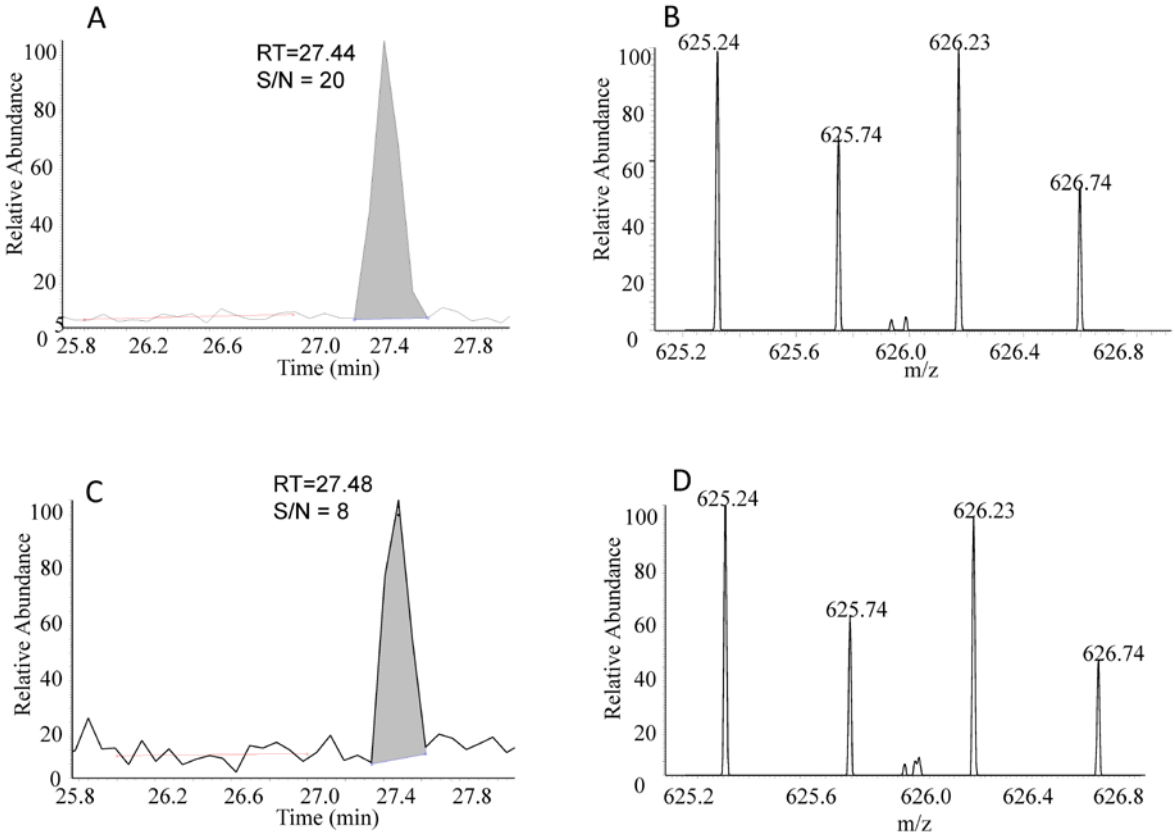


Figure S14. MS LOD Peaks of the Brominated Coumarin Triazole. MS peak of the penultimate lowest point (A) and the corresponding precursor ion spectrum (B); and MS peak of the lowest point (C) and the corresponding precursor ion spectrum (D) for LOD determination.

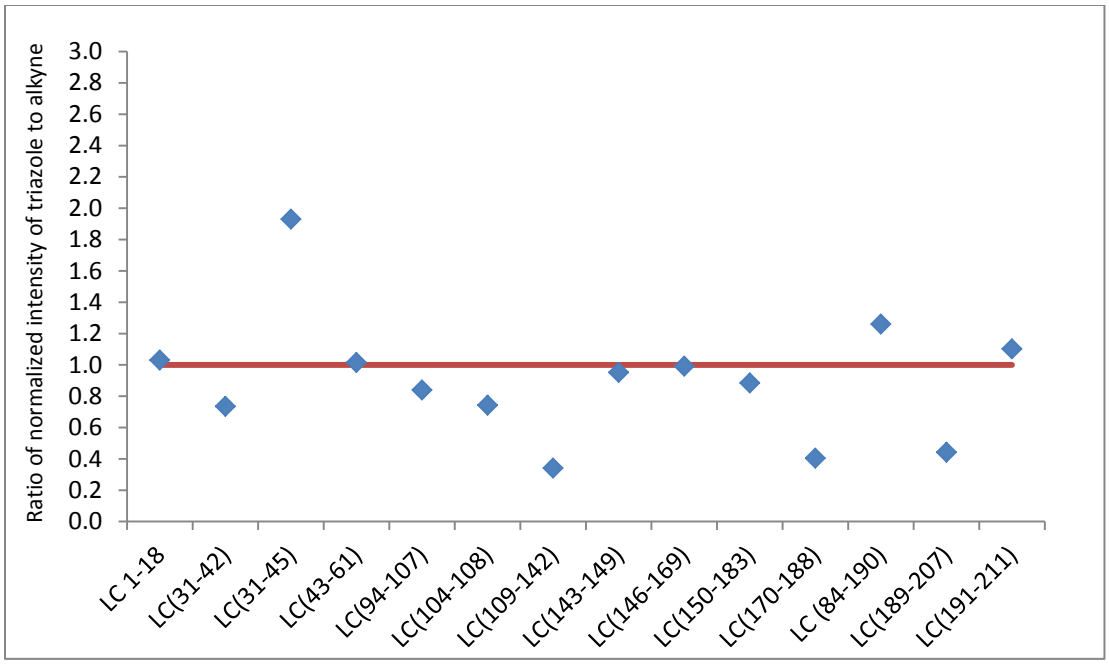


Figure S15. Effect of the Brominated Coumarin Azide on Mass Spectrometry Ionization Efficiency. Fourteen tryptic peptides from the mAb-alkyne and mAb-triazole light chain were selected for comparison. A light chain peptide (LC 19-24) VTITCR that was not modified by an alkyne served as the internal standard. The MS intensities of the peptide alkyne and triazole product were each divided by the intensity of the internal standard to obtain the normalized intensity. The ratio of the normalized intensity of the triazole to the alkyne was used to interpret the effect on ioniziation. If the ratio equaled 1.0 (red line), the brominated coumarin azide did not alter the peptide alkyne ionization efficiency. The majority of the ratios were between 0.8 and 1.2, indicating that the brominated coumarin azide did not significantly affect ionization efficiency. There was one peptide with a ratio higher than 1.2 and three with ratios lower than 0.8, suggesting other factors outside of the brominated coumarin azide influenced ionization.

Table S1. Effect of the Brominated Coumarin Azide on Ionization Efficiency of Charge States. The intensity of the higher charge state (z+1) and the lower charge state (z) for each triazole click product and alkyne was acquired, and the ratio of these intensities was obtained. From these ratios, the ratio of the triazole product to the alkyne was determined. The brominated coumarin azide altered the distribution of ionization toward higher charge states, but there was no change in the maximum charge state.

Peptide	Alkyne intensity	Triazole intensity	Charge state	Ratio of intensity of charge (z+1/z)		
				Alkyne	Triazole	Triazole to Alkyne
LC 1-18	2.27E+07	1.52E+07	2	0.025	0.094	3.82
	5.57E+05	1.43E+06	3			
LC 31-42	7.69E+06	3.70E+06	2	0.0048	0.067	14.15
	3.65E+04	2.49E+05	3			
LC 31-45	1.83E+07	1.30E+07	2	0.087	1.05	12.07
	1.60E+06	1.37E+07	3			
LC 43-61	1.55E+07	7.81E+06	2	0.45	1.03	2.30
	6.94E+06	8.03E+06	3			
LC 94-107	7.78E+06	4.01E+06	2	0.025	0.16	6.56
	1.91E+05	6.45E+05	3			
LC 104-108	5.89E+06	1.69E+06	1	0.85	2.32	2.73
	5.01E+06	3.93E+06	2			
LC 109-142	1.73E+07	4.13E+06	3	0.086	0.076	0.89
	1.49E+06	3.15E+05	4			
LC 143-149	6.85E+06	1.23E+06	1	2.00	10.04	5.01
	1.37E+07	1.24E+07	2			
LC 146-169	4.63E+06	1.76E+06	2	0.71	2.09	2.97
	3.27E+06	3.69E+06	3			
LC 150-183	7.08E+06	3.98E+06	3	0.015	0.041	2.67
	1.10E+05	1.64E+05	4			
LC 170-188	4.84E+06	1.05E+06	2	0.020	0.33	16.21
	9.71E+04	3.40E+05	3			
LC 184-190	1.46E+07	1.51E+06	1	1.26	14.85	11.82
	1.83E+07	2.24E+07	2			
	1.06E+05	4.93E+06	3			
LC 189-207	9.86E+06	1.45E+06	2	0.78	2.72	3.49
	7.70E+06	3.95E+06	3			
LC 191-211	4.40E+07	1.60E+07	2	0.50	2.12	4.22
	2.21E+07	3.39E+07	3			
	2.14E+05	8.54E+05	4			

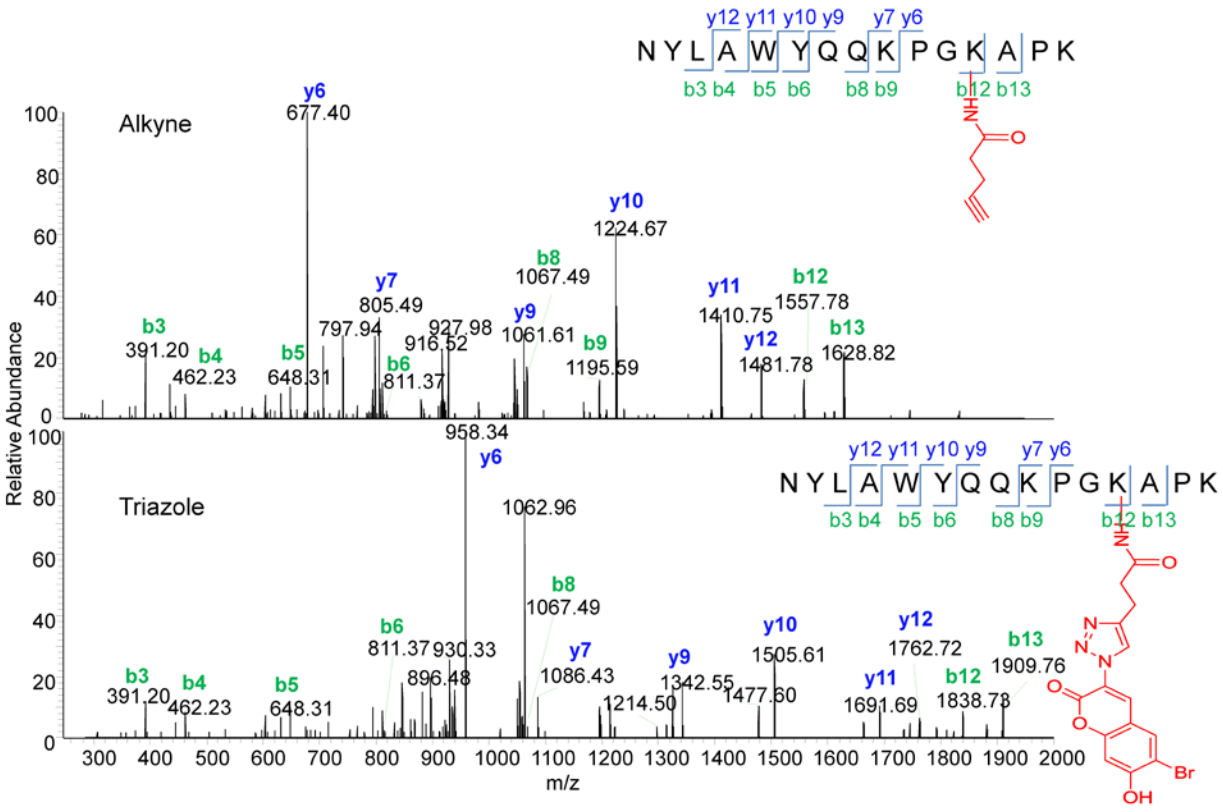


Figure S16. Effect of the Brominated Coumarin Azide on MS/MS Spectra. The y and b ions of the peptide alkyne NYLAWYQQKPGKAPK (top) and its click product (bottom) were similar, suggesting that the brominated coumarin azide did not affect the MS/MS fragmentation pattern.

Table S2. Retention Time and Eluting %B of Peptide Alkyne and Triazole Click Product. The alkyne and its corresponding brominated coumarin triazole click product were well-resolved chromatographically.

Peptide	Peptide alkyne		Triazole click product		Retention time difference (min)
	Retention time (min)	Eluting at %B	Retention time (min)	Eluting at %B	
LC (184-190)	13.85	4.16	46.71	12.20	32.86
LC (104-108)	32.22	8.65	64.72	16.60	32.5
LC (143-149)	45.45	11.89	73.52	18.75	28.07
LC (31-45)	64.11	16.45	82.72	21.00	18.61
LC (150-183)	79.9	20.31	94.83	23.96	14.93

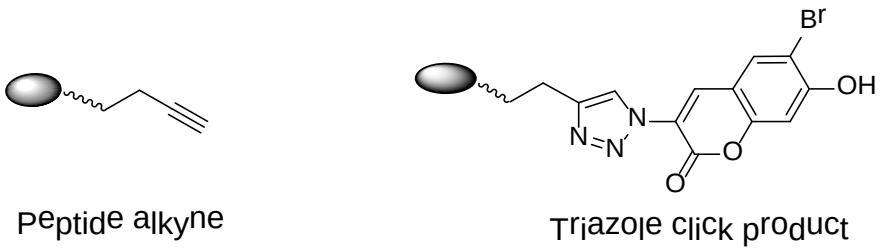


Figure S17. Structures of Peptide Alkyne and Triazole Click Product

4. Identification of Alkynes

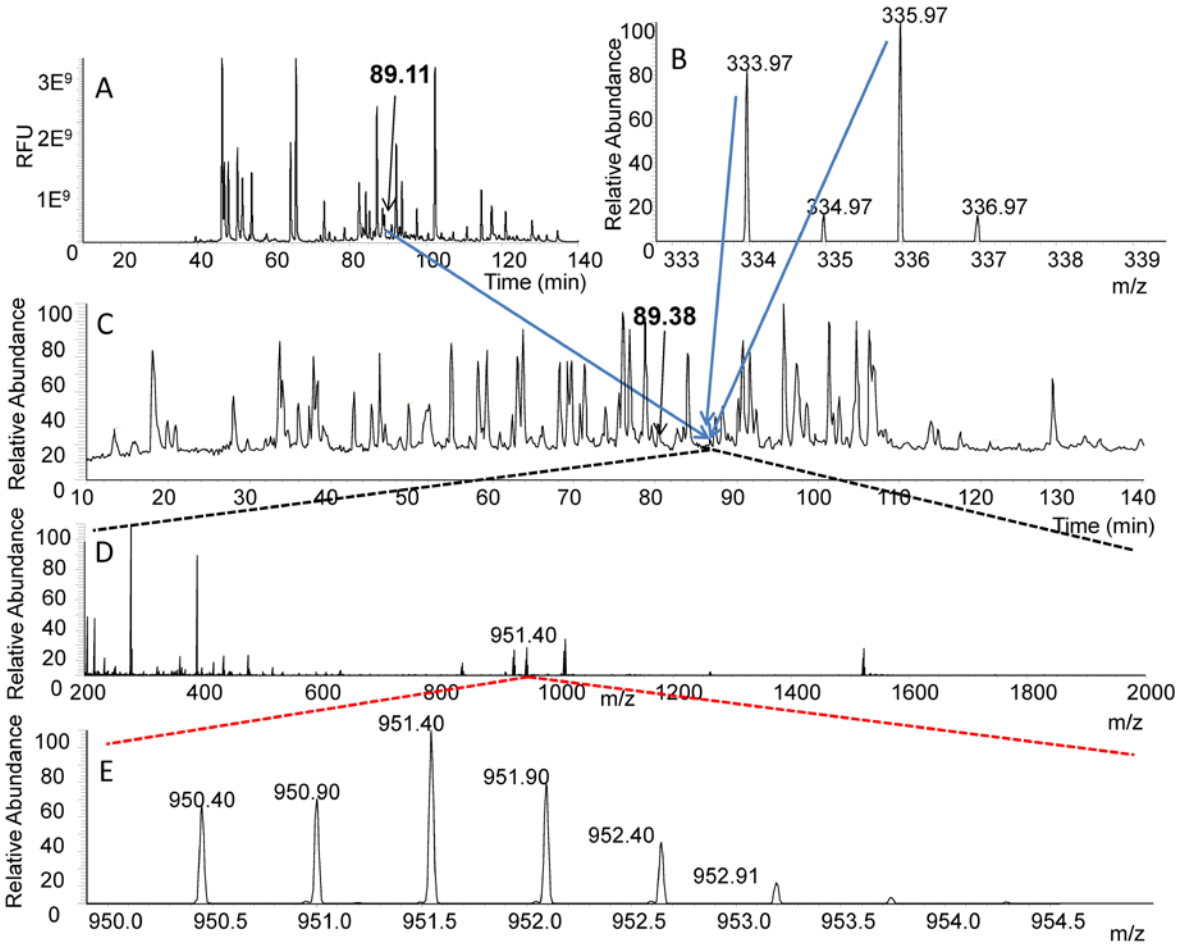


Figure S18. LC/Fluorescence/MS Analysis of Tryptic Peptides of mAb Triazole Click Product. Fluorescence chromatogram (A), Reporter ion pair (B), TIC (C), MS1 spectrum (D), and isotopic pattern of tagged peptide (E) (doubly charge MH2+ m/z = 950.40, equivalent to MH+= 1899.80, expected MH+=1899.79). The mass was subtracted by 360.97 Da to obtain the native peptide mass of 1539.01 which matched one missed-cleaved peptide in the mAb light chain residue 94-107, APYTFGQGTKVEIK.

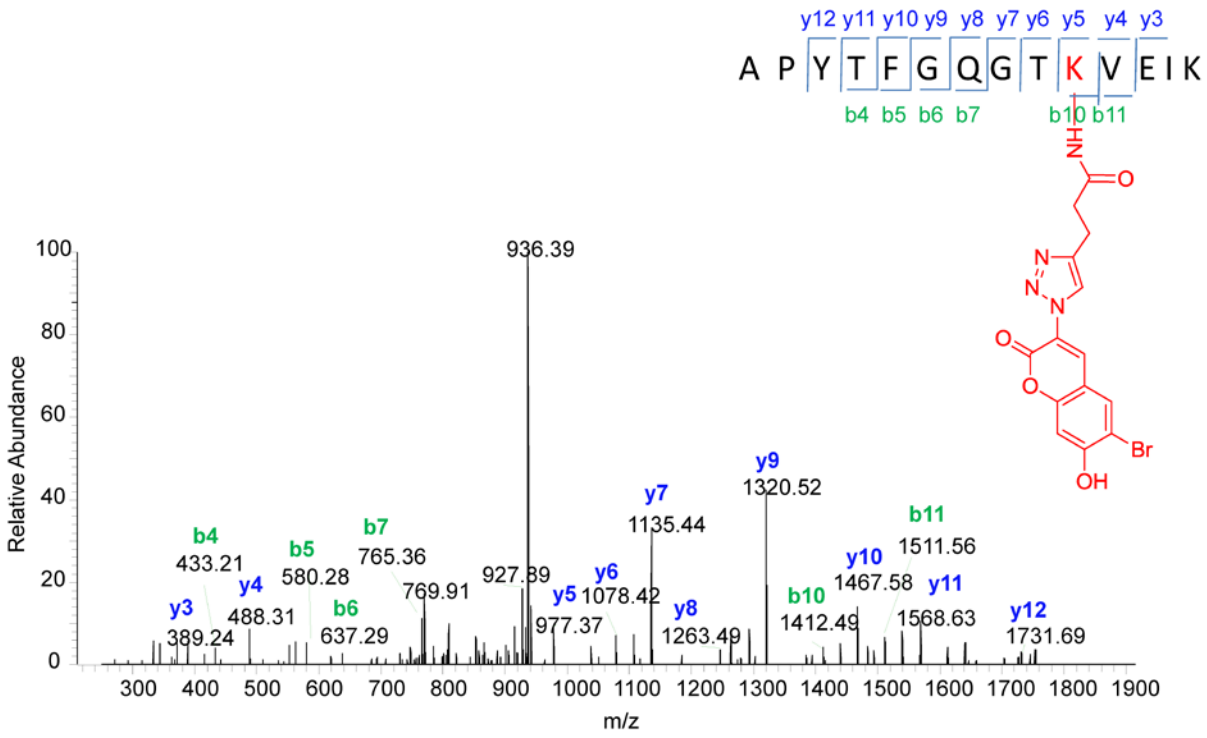


Figure S19. CID MS/MS of modified LC 94-107 APYTFGQGTKVEIK. In the b ion series, ions b4 through b7 were detected with expected mass and an additional mass of 360.97 Da was added to b10 (the lysine residue) and b11. In the y ion series, ions y3 and y4 were detected, and an additional mass of 360.97 Da was added to ions y5 (the lysine residue) through y12. MS/MS confirmed that K103 was modified.

Table S3. Detection of Modified Peptides in mAb Light Chain. Every lysine and the N-terminus were modified by the alkyne.

Fluorescence RT (min)	Isotopic Precursor Ion	Charge State	MH+1	MH+1 Without tag	Modified Peptide
89.26	928.88	2	1856.76	1495.76	LC (31-42)
82.56	718.31	3	2152.93	1791.93	LC (31-45)
121.03	1167.05	2	2333.1	1972.1	LC (43-61)
89.45	950.4	2	1899.8	1538.8	LC (94-107)
64.62	503.19	2	1005.38	644.38	LC (104-108)
149.22	1022.47	4	4086.88	3725.88	LC (109-142)
73.38	625.24	2	1249.48	888.48	LC (143-149)
86.58	1519.63	2	3038.26	2677.26	LC (146-169)
94.68	1327.57	3	3980.71	3619.71	LC (150-183)
99.06	1235.5	2	2470	2109	LC (170-188)
46.58	626.21	2	1251.42	890.42	LC (184-190)
74.61	835.02	3	2503.06	2142.06	LC (189-207)
87.15	914.72	3	2742.16	2381.16	LC (191-211)
92.34	1120.44	2	2239.88	1878.88	LC N-terminal

Table S4. Detection of Modified Peptides in mAb Heavy Chain. Lysines (18 out of 31) and the N-terminus were modified by the alkyne.

RT (min)	Isotopic Precursor Ion	Charge state	MH+1	MH+1 without tag	Modified Peptide
48.28	431.65	2	862.3	501.3	HC (293-296)
47.29	426.64	2	850.28	489.28	HC (215-218)
47.33	510.18	2	1017.36	656.36	HC (343-348)
50.75	550.17	2	1097.34	736.34	HC (325-330)
52.04	546.15	2	1089.3	728.3	HC (322-326)
53.95	663.71	2	1324.42	963.42	HC (219-226)
54.35	482.17	2	961.36	600.36	HC (218-222)
65.97	591.23	2	1179.46	818.46	HC (414-420)
84.39	814.36	2	1627.72	1266.72	HC (327-338)
93.82	815.87	2	1628.74	1267.74	HC (331-342)
97.76/98.07	1065.44	2	2127.86	1766.86	HC (73-87)
102.56	1056.45	2	2109.9	1748.9	HC (360-374)
102.79	993.93	2	1985.84	1624.84	HC N-terminal (1-16)
114.71	951.76	3	2851.27	2490.27	HC (126-151)
117.27	1234.53	3	3698.59	3337.59	HC (223-252)
121.89	1295.09	2	2588.18	2227.21	HC (306-324)
127.92	1070.81	3	3207.43	2846.43	HC (227-252)
134.65	1769.83	4	7075.32	6714.32	HC (152-214)

Table S5. Identified Unexpected Alkyne Species

Undefined Species	**Formation Mechanism**
HC (126-137) GPSVFPLAPSSK	Serine 136 in HC (126-137) GPSVFPLAPS**S**K
LC (184-189) ADYEKH	Cleavage between H and K in LC (184-190) ADYEK**H/K**
LC (191-200) SSPVTKSFNR	Cleavage between L and S in LC (191-211) VYACEVTHQG**L/S**SPVTKSFNR
LC (191-201) GLSSPVTKSFNR	Cleavage between Q and G in LC (191-211) VYACEVTH**Q/G**LSSPVTKSFNR
HC (73- 84) DNAKNSLYLQMN	Cleavage between N and S in HC (73- 87) DNAKNSLYLQM**N/S**LR

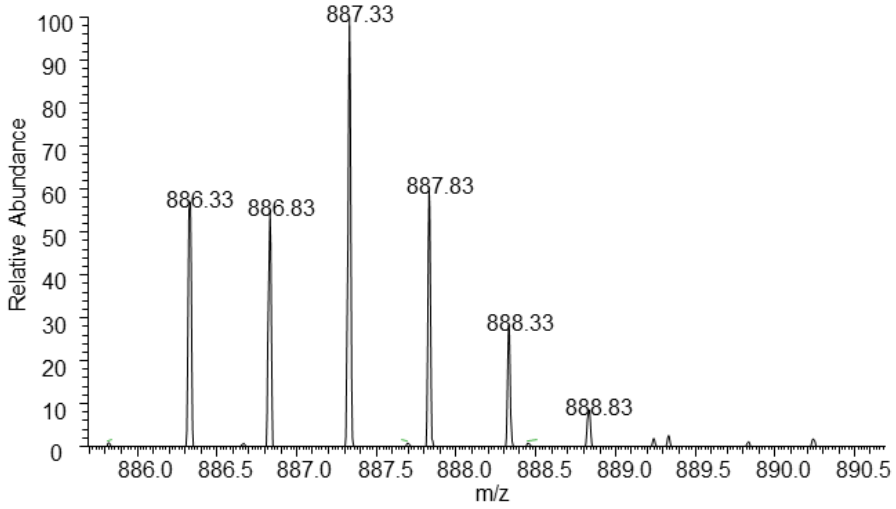


Figure S20. Precursor ion of HC (73-87) DNAKNSLYLQMN from cleavage between N and S in DNAKNSLYLQMNSLR. Lysine 76 was modified.

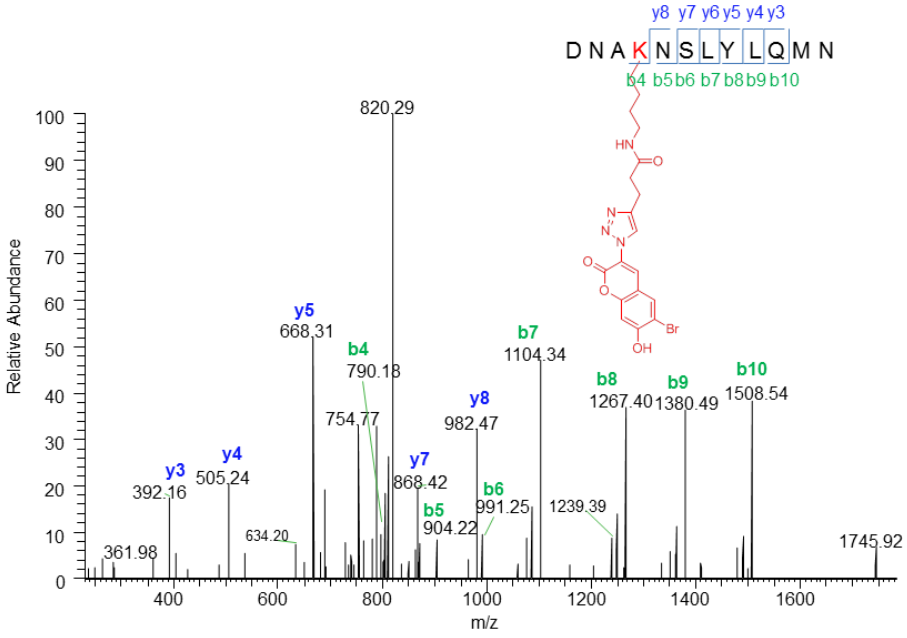


Figure S21. CID MS/MS of HC (73- 87) DNAKNSLYLQMN from cleavage between N and S in DNAKNSLYLQMNSLR. Lysine 76 was modified as D, N, and A were unlikely to be modified based on the chemistry of the reaction.

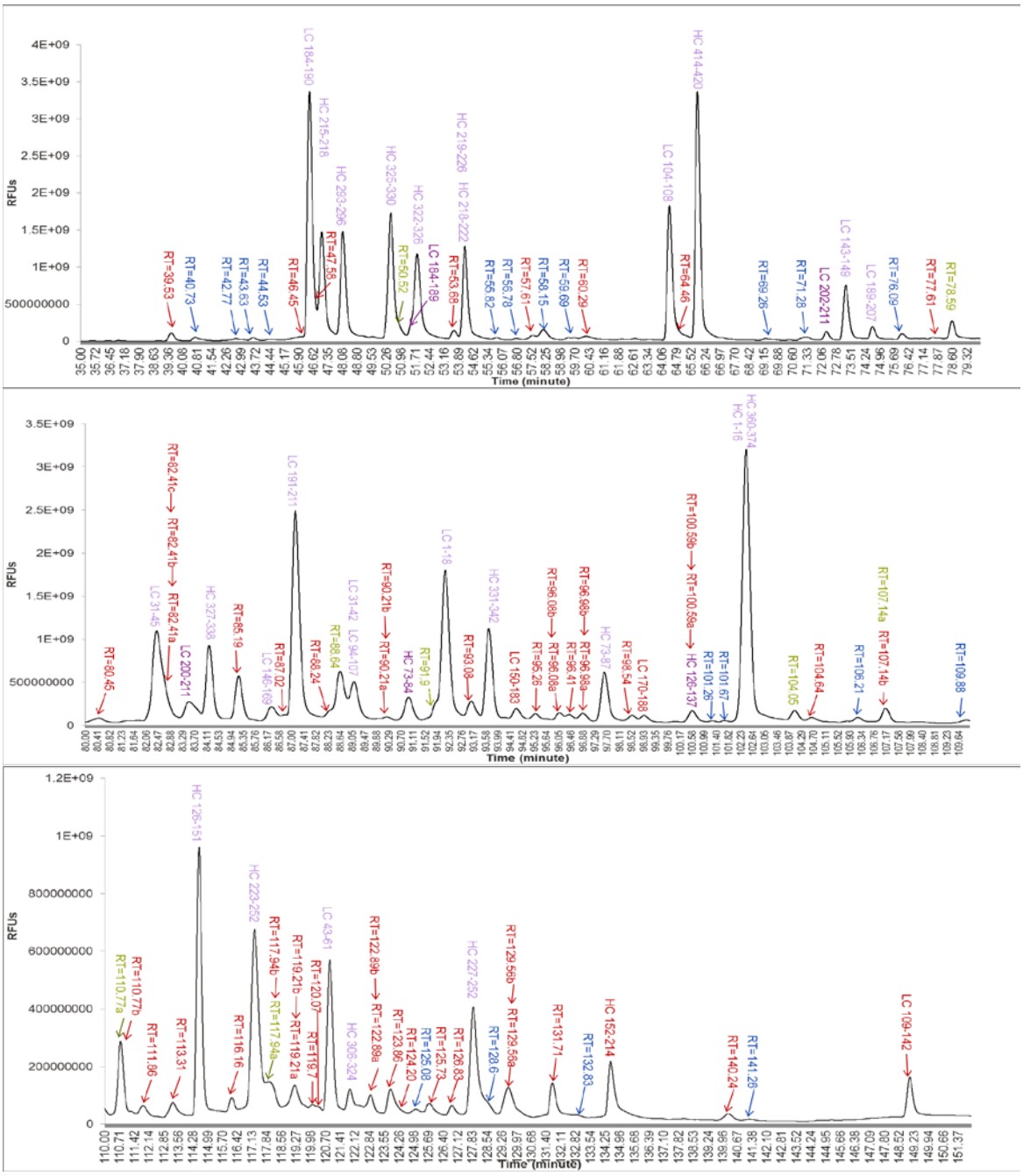


Figure S22. Fluorescent Peak Assignment of Modified Species. Top (RT 35-80 minutes); middle (RT 80 – 110 minutes); bottom (RT 110 – 150 minutes). Purple: detected by Byonic algorithm (33); green: detected only by reporter ions (8); red: detected only by isotopic signatures (52); blue: detected only fluorescence (19).

Table S6. Detection of Species by Various Methods

Species	MS/MS based Byonic algorithm	Reporter ion pair search in the MS/MS	Parent ion with isotopic signature	Fluorescence
LC #40 (184-190)	x	x	x	x
LC #32 (104-108)	x	x	x	x
LC #36 (143-149)	x	x	x	x
LC #41 (189-207)	x	x	x	x
LC #26 (31-45)	x	x	x	x
LC #37 (146-169)	x	x	x	x
LC #42 (191-211)	x	x	x	x
LC #4 (31-42)	x	x	x	x
LC #31 (94-107)	x	x	x	x
LC N-terminal (1-18)	x		x	x
LC #38 (150-183)			x	x
LC #39 (170-188)			x	x
LC #27 (43-61)	x	x	x	x
LC #34 (109-142)			x	x
HC #22 (293-296)	x	x	x	x
HC #54 (215-218)	x	x	x	x
HC #70 (343-348)	x	x	x	x
HC #66 (325-330)	x	x	x	x
HC #65 (322-326)	x	x	x	x
HC #56 (219-226)	x	x	x	x
HC #55 (218-222)	x	x	x	x
HC #77 (414-420)	x	x	x	x
HC #67 (327-338)	x		x	x
HC #68 (331-342)	x	x	x	x
HC #47 (73-87)	x	x	x	x
HC #73 (360-374)	x	x	x	x
HC N-terminal (1-16)	x		x	x
HC #51 (126-151)	x	x	x	x
HC #57 (223-252)	x	x	x	x
HC #64 (306-324)	x		x	x
HC #18 (227-252)	x	x	x	x
HC #13 (152-214)			x	x
LC (184-189)	x	x	x	x
LC (202-211)	x	x	x	x

Table S6. Detection of Species by Various Methods (Continued)

Species	MS/MS based Byonic algorithm	Reporter ion pair search in the MS/MS	Parent ion with isotopic signature	Fluorescence
LC (200-211)	x	x	x	x
HC (73-84)	x		x	x
HC (126-137)	x	x	x	x
RT 39.53			x	x
RT 40.73				x
RT 42.77				x
RT 43.63				x
RT 44.53				x
RT 46.45			x	x
RT 47.58			x	x
RT 50.52		x	x	x
RT 53.68			x	x
RT 55.82				x
RT 56.78				x
RT 57.61			x	x
RT 58.15				x
RT 59.69				x
RT 60.29			x	x
RT 64.46			x	x
RT 69.26				x
RT 71.28				x
RT 76.09				x
RT 77.61			x	x
RT 78.59		x	x	x
RT 80.45			x	x
RT 82.41a			x	x
RT 82.41b			x	x
RT 82.41c			x	x
RT 85.19			x	x
RT 87.02			x	x
RT 88.64		x	x	x
RT 88.24			x	x
RT 90.21a			x	x
RT 90.21b			x	x

Table S6. Detection of Species by Various Methods (Continued)

Species	MS/MS based Byonic algorithm	Reporter ion pair search in the MS/MS	Parent ion with isotopic signature	Fluorescence
RT 91.9		x	x	x
RT 93.08			x	x
RT 95.26			x	x
RT 96.08a			x	x
RT 96.08b			x	x
RT 96.41			x	x
RT 96.98a			x	x
RT 96.98b			x	x
RT 98.54			x	x
RT 100.59a			x	x
RT 100.59b			x	x
RT 101.26				x
RT 101.67				x
RT 104.05		x	x	x
RT 104.64			x	x
RT 106.21				x
RT 107.14a		x	x	x
RT 107.14b			x	x
RT 109.88				x
RT 110.77a		x	x	x
RT 110.77b			x	x
RT 111.86			x	x
RT 113.31			x	x
RT 116.16			x	x
RT 117.94a		x	x	x
RT 117.94b			x	x
RT 119.21a			x	x
RT 119.21b			x	x
RT 119.7			x	x
RT 120.07			x	x
RT 122.89a			x	x
RT 122.89b			x	x
RT 123.86			x	x
RT 124.20			x	x

Table S6. Detection of Species by Various Methods (Continued)

Species	MS/MS based Byonic algorithm	Reporter ion pair search in the MS/MS	Parent ion with isotopic signature	Fluorescence
RT 125.08				x
RT 125.73			x	x
RT 126.83			x	x
RT 128.60				x
RT 129.56a			x	x
RT 129.56b			x	x
RT 131.71			x	x
RT 132.83				x
RT 140.24			x	x
RT 141.26				x

Table S7. Relative Quantitation of Modified Species by Fluorescence

Modified Peptide	Fluorescence Peak Area (RFU*min)	% to the Most Prevalent Peak
LC (31-42)	1.14E+10	18%
LC (31-45)	3.08E+10	49%
LC (43-61)	1.22E+10	20%
LC (94-107)	1.14E+10	18%
LC (104-108)	3.66E+10	59%
LC (109-142)	3.30E+09	5%
LC (143-149)	1.51E+10	24%
LC (146-169)	3.15E+09	5%
LC (150-183)	5.26E+09	8%
LC (170-188)	2.61E+09	4%
LC (184-190)*	**6.23E+10**	**100%**
LC (189-207)	4.72E+09	8%
LC (191-211)	4.82E+10	77%
LC N-terminal	3.94E+10	63%
HC (293-296)	3.04E+10	49%
HC (215-218)	3.06E+10	49%
HC (343-348)	3.21E+10	52%
HC (325-330)	3.96E+10	64%
HC (322-326)	3.25E+10	52%
HC (219-226)	3.27E+09	5%
HC (218-222)	2.48E+10	40%
HC (414-420)	5.74E+10	92%
HC (327-338)	1.77E+10	28%
HC (331-342)	2.01E+10	32%
HC (73-87)	1.23E+10	20%
HC (360-374)/HC N-terminal (1-16) #	6.29E+10	101%
HC (126-151)	1.71E+10	27%
HC (223-252)	1.87E+10	30%
HC (306-324)	1.66E+09	3%
HC (227-252)	1.19E+10	19%
HC (152-214)	5.53E+09	9%
HC (126-137) (serine modification)	4.55E+09	7%
LC (191-200) (cleavage)	3.15E+09	5%
LC (191-201) (cleavage)	8.76E+09	14%
HC (73- 84) (cleavage)	8.41E+09	13%

*: LC (184-190) was the most prevalent peak and used for relative quantitation.

#: HC (360-374) and HC N-terminal (1-16) coeluted and value reported is the total.

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(2) Abitan, H., Bohr, H. and Buchhave, P. (2008) Correction to the Beer-Lambert-Bouguer law for optical absorption. *Appl Opt*, *47*, 5354-5357.

(3) Locatelli, M., Carlucci, G., Genovese, S., Curini, M. and Epifano, F. (2011) Use of HPLC in the Determination of the Molar Absorptivity of 4'-Geranyloxyferulic Acid and Boropinic Acid. *Chromatographia*, *73*, 889-896.