A Protein Functionalization Platform Based On Selective Reactions at Methionine Residues.

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Supplementary Information

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General Information

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at ambient temperature on a Bruker AM 400 (400 MHz) or an Avance 500 (500 MHz) spectrometer. Chemical shifts (δ) are reported in ppm and quoted to the nearest 0.01 ppm relative to the residual protons in chloroform-d (7.26 ppm) or acetonitrile-d_3 (1.94 ppm) and coupling constants (J) are quoted in Hertz (Hz). Data are reported as follows: Chemical shift (, multiplicity, coupling constants, number of protons). Multiplicity is reported according to the following convention: s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, sext = sextet, sp = septet, m = multiplet, br = broad. Where coincident coupling constants have been observed, the apparent (app) multiplicity of the proton resonance has been reported. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded at ambient temperature on a Bruker AM 400 (100 MHz) or an Avance 500 (125 MHz) spectrometer. ^{19}F NMR spectra were recorded at 376 MHz on a Bruker AVIII spectrometer. Chemical shifts for ^{19}F NMR are reported with respect to fluorobenzene (C_6H_5F; δF = −115.3 ppm in acetonitrile-d_3)[1] added as internal standard. Chemical shift (δ) was measured in ppm and quoted to the nearest 0.1 ppm relative to the residual solvent peaks in chloroform-d (77.16 ppm) or acetonitrile-d_3 (1.32 ppm). Infrared (IR) spectra were recorded on a Perkin Elmer 1FT-IR Spectrometer fitted with an ATR sampling accessory as either solids or neat films, either through direct application or deposited in chloroform, with absorptions reported in wavenumbers (cm^{-1}) Analytical thin layer chromatography (TLC) was performed using pre-coated Merck glass backed silica gel plates (Silicagel 60 F254). Flash column chromatography was performed using Material Harvest silica gel (230–400 mesh) under a positive pressure of compressed air unless otherwise stated. Visualization was achieved using ultraviolet light (254 nm) or with basic potassium permanganate solutions as appropriate. X-ray crystallography was performed on a Nonius Kappa CCD at the Cambridge University Chemistry X-Ray Laboratory. Tetrahydrofuran, toluene, hexane, diethyl ether and dichloromethane were dried and distilled using standard methods. For reactions requiring degassed solvents, the solvents were sparged with a positive pressure of nitrogen for a minimum of one hour before use. High-resolution mass spectrometry was performed by the University of Cambridge, Department of Chemistry Mass Spectrometry Service. MS/MS spectra were obtained by the Protein and Nucleic Acids Facility at the University of Cambridge, Department of Biochemistry. Circular Dichroism experiments were performed on an Applied Photophysics Chirascan Circular Dichroism Spectrophotometer. We thank Dr. Marco Di Antonio for his assistance in acquiring CD spectra.

Reactions that did not involve protein modification were carried out using glassware that was dried under vacuum using a heat gun and run under an atmosphere of nitrogen unless otherwise stated. All reactions were monitored by TLC or LCMS (Shimadzu LCMS 2020, method: 5-95%B over 3.5 min, then hold 0.5 min, then 95-5%B over 0.5 min min, hold 0.5 min, 0.7 mL/min) as appropriate. 2,4-difluorophenyl- λ^3-iodanediyl diacetate[2], (*o*-tolyl)-λ^3-iodanediyl diacetate[2], N,N'-ditosyl hydrazine[3], propargyl diazoacetate[3], α-diazobenzophenone[3], 5(6)-carboxyfluorescein diacetate[4] and methyl (*tert*-butoxycarbonyl)-*L*-methioninate[5] and iodonium salt **1a**[6] were prepared according to literature procedures. Exenatide•OAc, Teriparatide•OAc, and Aviptadil•OAc were purchased from Selleckchem; tetracosactide•OAc was purchased from Alpha-Diagnostics. Met-Gly, Aprotinin (from bovine lung, 3–7 TIU/mg, received containing carrying amounts of methionine sulfoxide), Ubiquitin (from bovine erythrocytes, BioUltra grade), Thioredoxin (from *E. coli*, recombinant, >3 units/mg), and α-

Lactalbumin (type III, >85%) were purchased from Sigma-Aldrich. All peptides and proteins were used without any additional purification. Azide **17**, was purchased from Sigma-Aldrich and used as received. All other reagents were acquired from commercial sources and used as received. Protein modification reactions were monitored and outcomes assessed using either a Shimadzu LCMS-2020 (solvents: A: 0.05% Formic acid in H_2O B: 0.05% formic acid in 95:5 CH_3CN:H_2O) equipped with an APCI probe, or a Waters XEVO GII-S Q-TOF equipped with an ESI probe (solvent system: A: 0.1% formic acid in H2O, B: 0.1% formic acid in 95:5 CH_3CN:H_2O). Conversions and product ratios were estimated either using total ion count (TIC, Shimadzu LCMS-2020), or MaxEnt software[7] (Waters XEVO). Note: Due to the formation of cationic sulfur, the MaxEnt software underestimates the mass of the parent protein+label peak by 1 hydrogen.

For MSMS analysis, frozen peptide stocks were thawed on ice followed by gentle vortexing. Each peptide was diluted to a final concentration of 1µM in 50% v/v acetonitrile, 0.1% v/v acetic acid for mass spectrometry analysis.
Peptide ions were generated by electrospray ionisation (ESI) using a TriVersa NanoMate (Advion BioSciences, Inc., Ithaca, NY) from a 10µl injection with an applied spray voltage of 1.55kV and a gas pressure of 0.7psi. Ions generated by ESI were introduced directly into an Orbitrap Fusion tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). An accurate mass full scan from 100-2000 *m/z* was performed at a resolution of 120,000 (full width at half-maximum, FWHM) with data acquisition for 1 minute using the mass spectrometer instrument control software, Orbitrap Tune Application 2.0. Peptide species charge states in the collected raw spectra were determined by manual interpretation using the theoretical monoisotopic mass**.** The most intense observed charge state for each peptide was selected for higher energy collisional dissociation (HCD) and electron transfer dissociation (ETD) fragmentation.
Precursor ion isolation was performed in the mass selecting quadrupole with an isolation window of 2 *m/z*. The precursor automatic gain control (AGC) target value was 1.5e5 with a maximum injection time of 200msec. The normalised collision energy (NCE) for HCD fragmentation was optimised to 30%. ETD ion reaction time was set to 100msec with an anion AGC of 2.5e5 and a maximum injection time of 200msec. Supplemental collisional activation (SA) in EThcD was optimised to 25%. Spectra for both fragmentation methods were acquired over a mass range of 100-4000 *m/z.* Each accurate mass full scan and subsequent MS2 fragmentation strategy was analysed in duplicate.
Peptide MS2 spectra from HCD and ETD fragmentation approaches were loaded into mMass 5.5 (http://www.mmass.org) for processing and deconvolution [1]. Observed peptide fragments were matched to theoretical fragment predictions for each peptide allowing a maximum charge state of 5 and a match tolerance of 20ppm. The Lorikeet spectrum viewer (http://code.google.com/p/lorikeet/) was used to view combined ETD and HCD MS2 spectra annotated with y, b, c and z fragment ions. Fragmentation data were further validated by manual inspection of the spectra.

References

[1] Strohalm M, Hassman M, Košata B, Kodíček M (2008)
mMass Data Miner: an Open Source Alternative for Mass Spectrometric Data Analysis.

Rapid Commun Mass Spec 22 (6), 905-908
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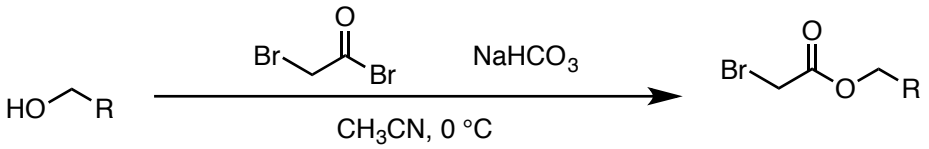
General Synthetic Procedures.

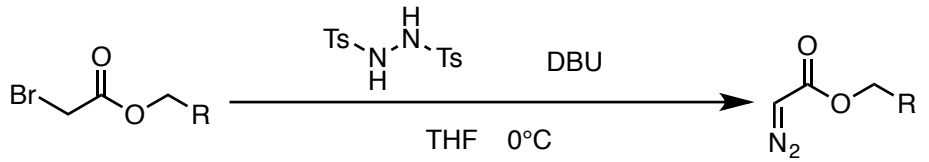
Synthesis of Iodonium Salts 1.

General Procedure A: Synthesis of substituted α-bromo esters.



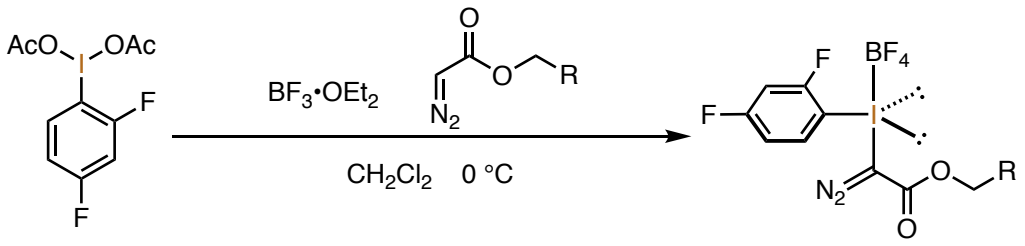
The synthesis of substituted α-bromoesters was achieved by adapting the procedure of Fukyama[3] as follows: A round-bottomed flask equipped with a magnetic stirrer and side-armed inlet adaptor was charged with sodium bicarbonate (5 eq.) and then evacuated and refilled with N_2. To the flask was added the desired alcohol (1 eq) and acetonitrile (0.15M in alcohol), and the resulting mixture chilled in an ice bath. To the stirring solution was added bromoacetyl bromide (1.5 eq), and the resulting mixture allowed to stir for 1-1.5 hours. The reaction mixture is quenched with $NaHCO_3$ (aq) and diluted with either diethyl ether or methylene chloride. The mixture is transferred to a seperatory funnel and the layers separated. The organic layer was washed twice with $NaHCO_3$ (aq) and once with brine, dried over magnesium sulfate, filtered, and the solvent removed using a rotary evaporator to yield the desired α-bromoester.

General Procedure B: Synthesis of substituted α-diazo esters from α-bromoesters



The synthesis of substituted α-diazoesters was achieved by adapting the procedure of Fukyama[3] as follows: A round-bottomed flask equipped with a magnetic stirrer and side-armed inlet adaptor was charged with *N,N'*-ditosylhydrazine (1.5 eq.) and then evacuated and refilled with N_2. To the flask was added the desired α-bromoester (1 eq) and tetrahydrofuran (0.2M in α-bromoester), and resulting mixture chilled in an ice bath. To the stirring solution was added 1,8–diaza–[4.4.0]-bicyclo–undec–7–ene (3 eq) in a rapid dropwise fashion, and the resulting mixture allowed to stir for 1 hour. The reaction mixture is quenched with $NaHCO_3$ (aq) and diluted with either diethyl ether or methylene chloride. The mixture is transferred to a seperatory funnel and the layers separated. The aqueous layer is extracted with either diethyl ether or methylene chloride. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated onto silica gel using a rotary evaporator. Purification by column chromatography furnished the desired α-diazoester.

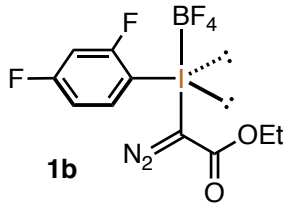
General Procedure C: Synthesis of iodonium salts from α-diazoesters.



A round-bottomed flask equipped with a magnetic stirrer and a sidearm inlet adaptor is heat-dried under vacuum. The flask was charged with freshly recrystallized (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (1 eq.) and is evacuated and refilled with N_2. To the flask was added methylene chloride (0.15M) via syringe, and the flask stirred vigorously and chilled in an ice bath. To the stirring solution was added $BF_3 \cdot OEt_2$ (1.1–1.3 eq) and the resulting solution was stirred for 5 minutes. To the flask was added the desired α-diazocarbonyl (1.1–1.3 eq) at a rapid dropwise rate, and the resulting solution stirred for 30 minutes at 0 °C. The solvent is then removed using a rotary evaporator. The resulting orange/red residue is triturated using a sonicator with mixtures of methylene chloride, diethyl ether, and 40-60 petroleum ether to yield the desired iodonium salt.

Safety Notice: Whilst we have prepared the following iodonium salts without incidence (nor have any issues been reported in previous literature), we have not prepared these reagents on a scale larger than 3 millimoles (ca. 1 gram), nor have we performed relevant calorimetry tests. Given the inherent chemical energy of these iodonium salts, we strongly recommend that users follow the appropriate safety procedures commensurate with preparing high-energy reagents.

(1-diazo-2-ethoxy-2-oxoethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate (1b)



1b

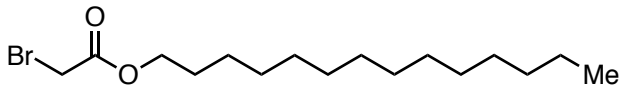
1b was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.94 g, 2.6 mmol), boron trifluoride diethyl etherate (0.42 mL, 3.0 mmol), and ethyl diazoacetate (0.41 mL, 87% purity, 3.0 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (5:20:75 CH_2Cl_2:40-60 petroleum ether:diethyl ether, 20 mL) afforded **1b** (1.00 g, 87% yield) as a yellow, free flowing, powder. Crystals suitable for X-ray diffraction were grown *via* slow diffusion of 40-60 petroleum ether into an ethyl acetate solution of **1b**. **1b** shows good stability (>3 months) when stored in a –20 °C freezer. **1b** displays the following stability profile in D_2O: 24hrs: 90% remaining, 48hrs: 69% remaining.

MP: 74 °C (decomp)

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.23 (app dt, J = 6.4, 9.0 Hz, 1H), 7.40 (app td, J = 2.7, 8.8 Hz, 1H), 7.25 (app td, J = 2.5, 8.6 Hz, 1H), 4.31 (q, J = 7.1 Hz, 2H), 1.28 (t, J = 7.1 Hz, 3H).

^{13}C-NMR (125 MHz, $CDCl_3$) δ: 166.9 (dd, *J* = 12.2, 258 Hz), 161.3 (dd, *J* = 14 Hz, 254 Hz), 160.4, 139.0 (d, *J* = 11 Hz), 115.4, (app dd, *J* = 2.8, 23 Hz), 106.2 (app t, *J* = 27 Hz, 1C), 96.5 (app d, *J* = 23 Hz), 64.3, 41.3 (s, br), 13.3.
^{19}F-NMR (376 MHz, CD_3CN) δ: –92.9 (d, *J* = 12 Hz), –100.0 (app d, *J* = 12 Hz), -151.3– -151.6 (m, br).
IR: 3086, 3065, 3037, 2988, 1698, 1682, 1598, 1587, 1474, 1426, 1371, 1270, 1192, 1155, 1117, 1056, 1041, 1001, 963, 876, 851, 822, 760, 732, 616.
HRMS-$(ESI)^+$ (m/z) Found ($M–BF_4$): 352.9604, Calc'd $C_{10}H_8O_2N_2F_2I$ requires 352.9593.

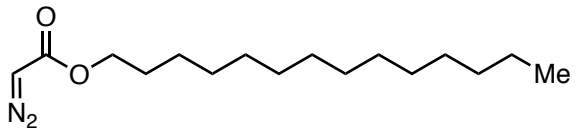
tetradecyl 2-bromoacetate (S-1)



S-1

S-1 was prepared according to General Procedure A using myristyl alcohol (6.50 g, 30 mmol), bromoacetyl bromide (7.8 mL, 90 mmol), sodium bicarbonate (12.9 g, 154 mmol) and methylene chloride (200 mL), with a reaction time of 2 hours. Methylene chloride was used in the workup. **S-1** was isolated (9.83 g, 97% yield) as a colorless oil that was of sufficient purity to be used directly in subsequent transformations. The structural characteristics of **S-1** are in agreement with previously reported values[9].
^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.17 (t, *J* = 7.6 Hz, 2H), 3.83 (s, 2H), 1.70-1.63 (app quint, *J* = 6.8 Hz, 2H), 1.39-1.19 (m, 22H), 0.88 (t, *J* = 6.9 Hz, 3H)
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 167.3, 66.5, 31.9, 29.7 (br, 5C), 29.6, 29.5, 29.4, 29.2, 28.4, 26.0, 25.6, 22.7.
IR: 2921, 2855, 1735, 1647, 1607, 1534, 1493, 1190, 1147, 1106, 888.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 335.1513, Calc'd $C_{16}H_{32}BrO_2$ requires 335.1586.

tetradecyl 2-diazoacetate (S-2)

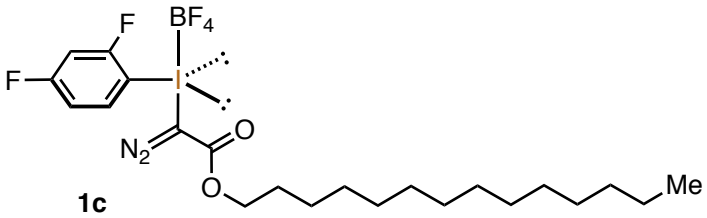


S-2

S-2 was prepared according to General Procedure B using **S-1** (1.68 g, 5.0 mmol), *N,N'*-ditosylhydrazine (2.58 g, 7.6 mmol), and 1,8-diazo-bicyclo-[5.4.0]-undec-7-ene (3.0 mL, 20 mmol), with a reaction time of 1 hour. Diethyl ether was used in the workup. Column chromatography (5% diethyl ether/40-60 petroleum ether) afforded **S-2** (0.976 g, 69%) as a yellow waxy solid that melts at room temperature.

R_f: 0.4 (20% Et_2O:40-60 petrol)
^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.72 (br, s), 4.15 (t, *J* = 6.7 Hz, 2H), 1.66-1.60 (m, 2H), 1.40-1.18 (m, 23H), 0.88 (t, *J* = 6.7 Hz, 3H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 167.1, 65.1, 46.1 (br), 31.9, 29.7 (br, 5C), 29.6, 29.5, 29.4, 28.8, 25.8, 22.7, 14.1.
IR: 2922, 2853, 2107, 1694, 1466, 1396, 1354, 1238, 1181, 1031 (br), 739, 721.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 283.2362, Calc'd $C_{16}H_{31}O_2N_2$ requires 283.2386.

(1-diazo-2-oxo-2-(tetradecyloxy)ethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate. (1c)

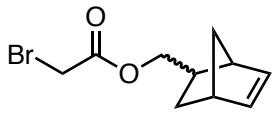


1c

1c was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.36 g, 1.0 mmol), boron trifluoride diethyl etherate (0.16 mL, 1.3 mmol), and **S-2** (0.37 g, 1.3 mmol), with a reaction time of 30 minutes. To the reaction solution was added water and CH_2Cl_2, and the layers were separated. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were carefully filtered over cotton and the solvent removed using a rotary evaporator. The resulting orange oil was dissolved in 40-60 petroleum ether (18 mL), and the solution was chilled in an acetone bath that was maintained between –30 to –40 °C, resulting in the formation of a yellow oil. After the oil had settled, the 40-60 petroleum ether was carefully decanted. The resulting mixture was warmed to room temperature and 40-60 petroleum ether (10 mL) was added, and the chilling process was repeated twice. The resulting yellow mixture was allowed to warm to room temperature, and any excess petroleum ether was removed using a rotary evaporator, yielding **1c** as an orange oil of 90% purity (0.29 g, 45%). While the carbon possessing the diazo moiety was not observable by ^{13}C-NMR, the IR stretch[8] at 2105 cm^{-1} as well as the correct mass being observed confirms the presence of the diazo group.

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.18 (ddd, *J* = 5.8, 6.8, 9.0 Hz, 1H), 7.34 (app td, *J* = 2.8, 8.8 Hz, 1H), 7.22-7.15 (m, 1H), 4.21 (t, *J* = 6.5 Hz, 2H), 1.68-1.57 (m, 2H), 1.37-1.22 (m, br, 22 H), 0.91 (t, *J* = 6.9 Hz, 3H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 166.3 (app d, *J* = 248 Hz), 161.7, 160.7 (app d, *J* = 252 Hz), 138.1, 115.0 (app d, *J* = 22 Hz), 105.9 (app t, *J* = 29 Hz), 67.6, 42.2 (br), 31.7, 29.4 (4C), 29.3, 29.2, 29.1, 28.9, 25.4, 22.4.
^{19}F-NMR (375 MHz, CD_3CN) δ: –93.9 (d, *J* = 12 Hz), –101.7 (d, *J* = 12 Hz), -151.7–-151.9 (m, br).
IR: 3097, 2922, 2853, 2105, 1699, 1587, 1525, 1467, 1423, 1390, 1271, 1187, 1033, 965, 850, 732.
HRMS-$(ESI)^+$ (m/z) Found ($M–BF_4$): 521.1487, Calc'd $C_{22}H_{32}O_2N_2F_2I$ requires 521.1471.

(1*S*,4*S*)-bicyclo[2.2.1]hept-5-en-2-yl)methyl 2-bromoacetate (S-3)



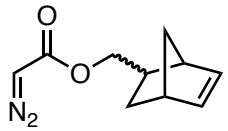
S-3

S-3 was prepared according to General Procedure A using norbornenol (1.245 g, 10 mmol, 1.7:1 mixture of *endo*– and *exo*–isomers), bromoacetyl bromide (2.6 mL, 30

mmol), and sodium bicarbonate (4.2 g, 50 mmol), with a reaction time of 1.5 hours. Diethyl ether was used in the workup. **S-3** was isolated (2.175 g, 88% yield) as a faint brown oil that was of sufficient purity (95%) to be used directly in subsequent transformations.

Peaks attributable to exo-S-3:
^{1}H-NMR (400 MHz, $CDCl_3$) δ : 6.14-6.09 (m, 2H), 4.27 (dd, *J* = 11.0, 6.5 Hz, 1H), 4.10 (dd, *J* = 11.4, 9.2 Hz, 1H), 3.88 (s, 2H), 2.88 (app s, 1H), 2.75 (app s, 1H), 1.83 (m, 1H), 1.42-1.37 (m, 1H), 1.36-1.25 (m, 3H), 1.19 (ddd, *J* = 11.8, 4.5, 3.5, 1H).
Peaks attributable to endo-S-3:
^{1}H-NMR (400 MHz, $CDCl_3$) δ : 6.19 (dd, *J* = 5.8, 3.0 Hz, 1H), 5.97 (dd, *J* = 5.8, 2.9 Hz, 1H), 3.98 (dd, *J* = 10.8, 6.6 Hz, 1H), 3.85 (s, 2H), 3.79 (dd, *J* = 11, 9.6 Hz, 1H), 2.93 (app s, 1H), 2.75 (app s, 1H), 2.50-2.40 (m, 1H), 1.88 (ddd, *J* = 12, 8.7, 3.8 Hz, 1H), 1.49 (ddd, *J* = 8.4, 4.8, 2.0 Hz, 1H), 1.31-1.26 (m, 1H), 0.59 (ddd, *J* = 11.7, 4.5, 2.5 Hz, 1H).
^{13}C-NMR (100 MHz, $CDCl_3$): 167.3, 167.2, 137.8, 137.0, 136.1, 132.1, 70.3, 69.7, 49.4, 44.9, 43.8, 43.6, 42.2, 41.6, 37.8, 37.6, 29.5, 28.9, 26.0 (2C).
IR: 2973, 2869, 1732, 1451, 1325, 1274, 1166, 1106, 987, 720.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 245.0171, Calc'd $C_{10}H_{13}O_2N_2$ requires 245.0177.

((1*S*,4*S*)-bicyclo[2.2.1]hept-5-en-2-yl)methyl 2-diazoacetate. (S-4)



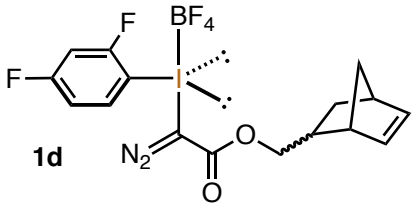
S-4

S-4 was prepared according to General Procedure B using **S-3** (2.45 g, 10 mmol), *N,N'*-ditosylhydrazine (5.11 g, 16 mmol), and 1,8-diazo-bicyclo-[5.4.0]-undec-7-ene (6.0 mL, 40 mmol), with a reaction time of 1 hour. Diethyl ether was used in the workup. Column chromatography (10% diethyl ether/40-60 petroleum ether) afforded **S-4** (1.39 g, 72%) as a yellow oil. **S-6** displays moderate stability in a –20 °C freezer, and should thus be used promptly after preparation.

R_f: 0.5 (20% ethyl acetate/40-60 petroleum ether)
^{1}H–NMR Peaks attributable to exo:
^{1}H-NMR (400 MHz, CD_3CN) δ : 6.13-6.08 (m, 2H), 4.96 (s, br, 1H), 4.20 (dd, *J* = 11.0, 6.5 Hz, 1H), 4.05 (dd, *J* = 11.3, 9.1 Hz, 1H), 2.83 (app s, 1H), 2.69 (app s, 1H), 1.72-1.65 (m, 1H), 1.38-1.34 (m, 1H), 1.32-1.18 (m, 3H).
^{1}H–NMR peaks attributable to endo:
^{1}H-NMR (400 MHz, CD_3CN) δ : 6.17 (dd, *J* = 5.8, 3.0 Hz, 1H), 5.95 (dd, *J* = 5.8, 2.9 Hz, 1H) 4.74 (s, br, 1H), 3.96 (dd, *J* = 10.8, 6.6 Hz, 1H), 3.75 (app t, *J* = 10 Hz, 1H), 2.88 (app s, 1H), 2.83 (app s, 1H), 2.46-2.35 (m, 1H), 1.85 (ddd, *J* = 11.9, 8.5, 3.8 Hz, 1H), 1.40 (ddd, *J* = 8.2, 4.9, 2.0 Hz, 1H), 1.30-1.27 (m, 1H), 0.56 (ddd, *J* = 11.9, 4.6, 2.5 Hz, 1H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 166.7 (br), 137.5, 136.9, 136.2, 132.1, 68.3, 67.7, 49.0, 45.9 (br), 44.6, 43.7, 42.1, 41.5, 38.2, 37.9, 29.0, 28.5.
IR: 3118, 2968, 2869, 2104, 1686, 1448, 1393, 1360, 1336, 1237, 1179, 1025, 990, 739, 720.

HRMS-(ESI)$^+$ (m/z) Found (M+H): 193.0965, Calc'd $C_{10}H_{13}O_2N_2$ requires 193.0972.

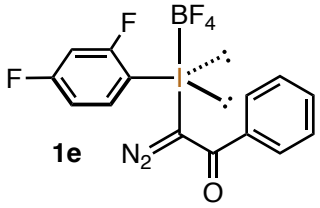
(2-(((1*R*,4*R*)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-1-diazo-2-oxoethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate.(1d) mixture of endo- and exo-isomers



1d was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.420 g, 1.2 mmol), boron trifluoride diethyl etherate (0.16 mL, 1.3 mmol), and **S-4** (0.248 g, 1.3 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (1:1 40-60 petroleum ether:diethyl ether, 30 mL, 10 mL (x 2), 40-60 petroleum ether, 10 mL, 20 mL, 30 mL) afforded **1d** (0.326 g , 54% yield) as an orange, sticky solid. While the carbon possessing the diazo moiety was not observable by ^{13}C-NMR, the IR stretch[8] at 2114 cm^{-1} as well as the correct mass being observed confirms the presence of the diazo group.

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.17 (app ddd, *J* = 5.8, 6.6, 9.1 Hz, 1H), 7.38-7.31 (m, 1H), 7.23-7.17 (m, 1H), 6.21 (dd, *J* =3.1, 5.7 Hz, 0.6 H), 6.16-6.09 (m, 0.6H), 5.93 (dd, *J* = 2.8, 6.0 Hz, 0.6H), 4.22 (ab q, *J* = 10.8, 68.9 Hz, 0.3H), 4.22 (ab q *J* = 10.8, 53 Hz, 0.3 H), 3.90 (ab q, *J* = 10.6, 91.3 Hz, 0.5H), 3.90 (ab q, *J* = 10.6, 74.2, 0.5 H) 2.85-2.78 (m, br, 3H), 2.64 (s, br, 0.6H), 2.44-2.37 (m, 1H), 1.87-1.80 (ddd, *J* = 3.9, 9.6, 11.7 Hz, 1H), 1.73-1.66 (m, 0.5 H), 1.43-1.37 (m, 1H), 1.33-1.15 (m, 3H), 0.54 (ddd, *J* = 2.6, 4.6, 11.7 Hz, 1H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 166.7 (dd, *J* = 11, 256 Hz), 161.1 (dd, 15, 156 Hz), 161.0, 160.8, 138.6 (d, *J* = 10 Hz), 137.8, 137.1, 136.0, 131.9, 115.3 (d, *J* = 24 Hz), 106.2 (app t, *J* = 26 Hz), 98.2 (br), 71.6, 71.0, 49.0, 44.5, 43.7, 43.4, 42.1, 41.5, 38.0, 37.6, 28.9, 28.3.
^{19}F-NMR (376 MHz, CD_3CN) δ: –93.7 (d, *J* = 12 Hz), –100.6 (d, *J* = 12 Hz), –151.8 (s), –151.9 (s).
IR: 3099, 2971, 2871, 2114, 1699, 1587, 1473, 1426, 1270, 1031 (br), 964, 850, 728.
HRMS-(ESI)$^+$ (m/z) Found (M–BF_4): 431.0045, Calc'd $C_{16}H_{14}O_2N_2F_2I$ requires 431.0063.

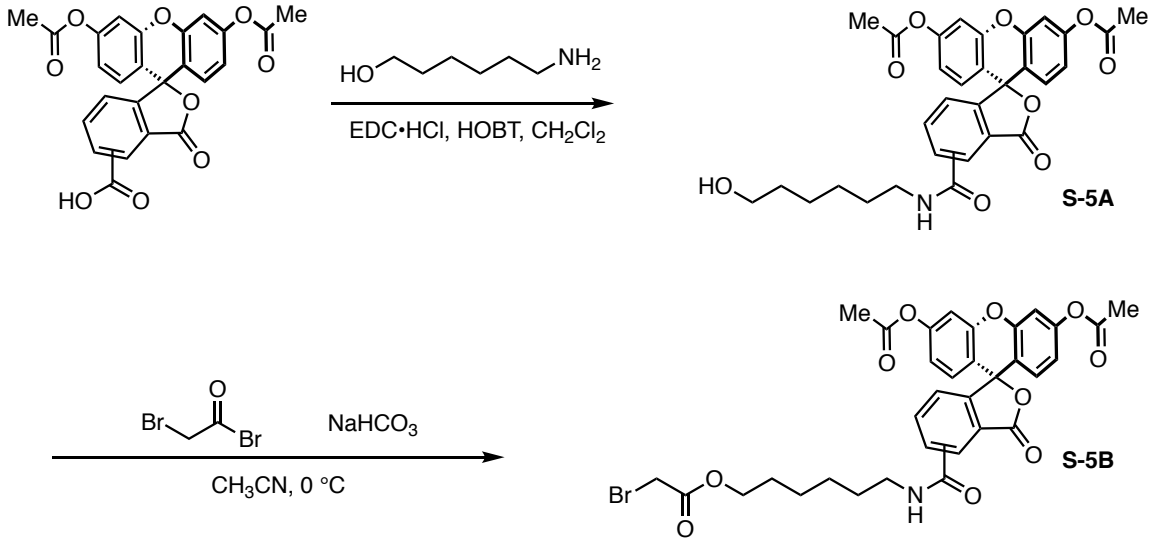
(1-diazo-2-oxo-2-phenylethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate. (1e)



1e was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.244 g, 0.68 mmol), boron trifluoride diethyl etherate (0.10 mL, 0.82 mmol), and α-diazobenzophenone[3] (0.121 g, 0.82 mmol), with a reaction time of 30 minutes. The reaction solution was then transferred to a flask containing a solution of 1:1 40-60 petroleum ether:diethyl ether (30 mL), and the resulting mixture was triturated using a sonicator, affording **1e** (0.263 g, 82%) as an orange, sticky semi-solid. **1e** should be stored in a –20 °C freezer and used within 1 month of preparation.

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.28 (app ddd, *J* = 5.8, 6.9, 9.1 Hz, 1H), 7.69-7.63 (m, 3H), 7.57-7.53 (m, 2H), 7.38 (app td, *J* = 2.8, 8.9 Hz, 1H), 7.26-7.21 (m, 1H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 182.1, 167.0 (dd, *J* = 12, 256 Hz), 161.7 (dd, *J* = 14, 256 Hz), 139.6 (d, *J* = 11 Hz), 133.7, 132.6, 129.3, 127.9, 115.5 (app d, *J* = 23 Hz), 106.3 (app t, *J* = 27 Hz), 95.4 (dd, *J* = 4, 24 Hz), 58.9.
^{19}F-NMR (376 MHz, CD_3CN) δ: –92.3 (d, *J* = 12 Hz), –100.0 (*J* = 12 Hz), –151.6—151.9 (m, br).
IR: 3099, 2971, 2871, 2114, 1699, 1587, 1473, 1426, 1270, 1031 (br), 964, 850, 728.
HRMS-$(ESI)^+$ (m/z) Found ($M–BF_4$): 384.9633, Calc'd $C_{14}H_8ON_2F_2I$ requires 384.9633.

5(6)-((6-(2-bromoacetoxy)hexyl)carbamoyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (S-5B)



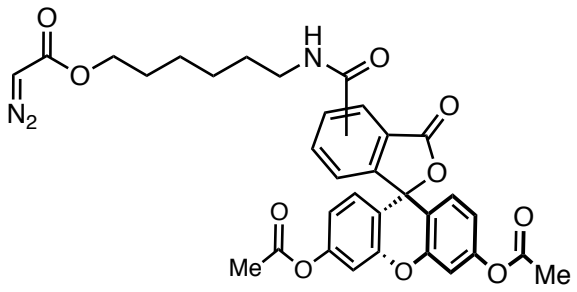
A round-bottomed flask equipped with a magnetic stirrer and a sidearm inlet adaptor is charged with 5(6)-carboxyfluorescein diacetate[4] (1.49 g, 3.2 mmol, 1:1 mixtures of regioisomers) and *N*–hydroxybenzotriazole (379 mg, 3.2 mmol) and is evacuated and refilled with N_2. To the flask was added methylene chloride (5 mL) and the resulting solution was stirred rapidly and chilled in an ice bath. To the flask was added *N*–ethyl–*N'*–dimethylaminopropyl carbodiimide (680 mg, 3.6 mmol) in a single portion, and the resulting mixture was stirred rapidly. After 7 minutes, the ice bath was removed, and the mixture stirred for a further 7 minutes. To the flask was added 6–amino–1–hexanol (529 mg, 3.9 mmol) in methylene chloride (6 mL) and the resulting mixture was stirred at room temperature for 4 hours. The reaction was quenched with NH_4Cl(aq), and the biphasic solution was transferred to a seperatory funnel where the layers were seperated. The aqeous layer was extracted once with methylene chloride. The combined organic layers were washed with 1M HCl(aq), $NaHCO_3$ (aq), and brine, then dried over $MgSO_4$, filtered and the solvent was removed using a rotary evaporator to yield crude **S-5A**.

The flask that contained crude **S-5A** was equipped with a magnetic stirrer and a sidearm inlet adaptor, was charged with $NaHCO_3$, and evacuated and refilled with N_2. To the flask was added acetonitrile (16 mL), and the mixture was stirred and chilled in a ice bath. To the stirring solution was added bromoacetyl bromide (0.85 mL, 9.7 mmol) in a rapid dropwise fashion, and the resulting solution stirred rapidly for 1 hour. The mixture was quenched with $NaHCO_3$(aq), diluted with methylene chloride, and the layers were seperated in a separatory funnel. The aqeuous layer is extracted with methylene chloride. The combined organic layers are then washed with $NaHCO_3$ (aq) and brine, dried with $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Column chromatography (20-40% ethyl acetate/ 40-60 petroleum ether) afforded a 1:1 regioisomeric mixture of **S-5B** (1.03 g, 47%) as a white semi-solid.

R_f: 0.2 (50% ethyl acetate:40-60 petroleum ether)
^{1}H-NMR (400 MHz, $CDCl_3$): 8.36 (app d, J = 1.0 Hz, 1H), 8.19 (dd, J = 8.2, 1.5 Hz, 1 H), 7.48-7.46 (m, 0.7H), 7.29 (app d, J = 8.2 Hz, 1 H), 7.12 (app d, J = 2.0 Hz, 2H), 7.09-7.06 (m, 1.3H), 6.85-6.78 (m, 7H), 6.45 (app t, J = 5.6 Hz, 1H), 6.34 (app t, J =

5.6 Hz, 0.7H), 4.19 (t, *J* = 6.5 Hz, 2H), 4.15 (t, *J* = 6.5 Hz, 1.5H), 3.85 (s, 2H), 3.80 (s, 1.4H), 3.54-3.46 (m, 2H), 3.43-3.36 (m, 1.5H), 2.35-2.30 (m, 10H), 1.74-1.63 (m, 3H), 1.49-1.41 (m, 3H), 1.41-1.34 (m, 2H).
^{13}C-NMR (100 MHz, $CDCl_3$): 169.0, 168.9, 168.4, 168.2, 167.4 (2C), 165.6 (2C), 155.1, 153.3, 152.3, 152.2, 151.6, 151.4, 141.6, 137.1, 134.8, 129.6, 129.1, 128.8, 128.2, 126.6, 125.7, 124.7, 123.1, 122.1, 118.0, 117.9, 115.8, 115.7, 110.6 (2C), 82.1, 81.7, 66.1, 66.0, 40.2 (2C), 29.4, 29.3, 28.3 (2C), 26.4, 26.3, 25.9 (2C), 25.4, 25.3, 21.1.
IR: 3072, 2934, 2856, 1757 (br), 1643, 1608, 1546, 1493, 1419, 1368, 1277, 1241, 1193, 1150, 1107, 1012, 993, 890.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 680.1111, Calc'd $C_{33}H_{31}O_{10}NBr$ requires 680.1126.

5(6)-((6-(2-diazoacetoxy)hexyl)carbamoyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (S-6)

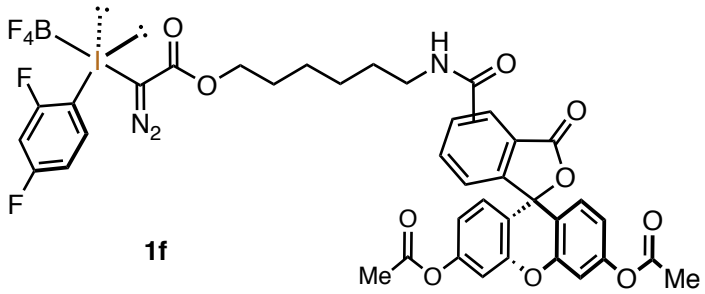


S-6

S-7 was prepared according to General Procedure B using **S-5B** (0.301 g, 0.4 mmol), *N,N'*-ditosylhydrazine (0.230 g, 0.7 mmol), 1,8-diazo-bicyclo-[5.4.0]-undec-7-ene (0.27 mL, 1.8 mmol), and a reaction time of 1 hour, after which acetic anhydride was added (0.2 mL). Dichloromethane was used in the workup. Column chromatography (30-50% ethyl acetate/40-60 petroleum ether) afforded **S-6** as an impure solid. The solid was triturated with ether, filtered, and the solvent removed using a rotary evaporator to afford **S-7** (0.111 g, 40% yield) as a yellow/green semi-solid.

R_f: 0.01 (50% ethyl acetate/40-60 petroleum ether)
^{1}H-NMR (400 MHz, CD_3CN) δ: 8.41 (app s, 0.9H), 8.20 (dd, *J* = 8.1, 1.5 Hz, 1H), 8.13-8.08 (m, 1.4H), 7.63 (app s, 0.7H), 7.37 (app d, *J* = 8.1 Hz, 1H), 7.32 (app t, *J* = 5.5 Hz, 1H), 7.19-7.12 (m, 3.9H), 6.97-6.87 (m, 7H), 5.00-4.89 (m, br, 1.25H), 4.15 (t, *J* = 6.5 Hz, 2H), 4.09 (t, *J* = 6.5 Hz, 1.4H), 3.44-3.38 (m, 2H), 3.32-3.25 (m, 1.4H), 2.29 (s, 10H), 2.18 (s, 6.9H), 1.72-1.48 (m, 6H), 1.47-1.39 (m, 3H), 1.31 (m, 2.7H).
^{13}C-NMR (100 MHz, CD_3CN) δ: 170.0 (2C), 169.1, 169.0, 166.1, 166.0, 155.6, 153.8, 153.5 (2C), 152.3, 142.9, 138.3, 135.6, 130.3, 130.2, 130.1, 128.8, 127.3, 126.1, 125.0, 124.5, 123.4, 119.3 (2C), 117.0 (2C), 111.4 (2C), 82.4, 65.4, 65.3, 46.7 (br), 40.5, 40.4, 29.9, 29.7, 29.4, 29.3, 27.2, 27.1, 26.1 (2C), 21.2.
IR: 3371, 3113, 2934, 2859, 2109, 1759, 1688, 1645, 1608, 1493, 1420, 1365, 1191, 1150, 1108, 1012, 993, 890.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 628.1910, Calc'd $C_{33}H_{30}O_{10}N_3$ requires 628.1926.

5(6)-((6-(2-diazo-2-((2,4-difluorophenyl)(tetrafluoro-λ^5-boraneyl)-λ^3-iodaneyl)acetoxy)hexyl)carbamoyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate. (1f) mixture of regioisomers

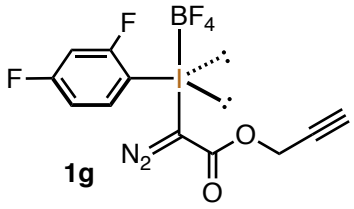


1f

1f was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.106 g, 0.30 mmol), boron trifluoride diethyl etherate (0.03 mL, 0.24 mmol), and **S-6** (0.154 mg, 0.24 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (1:4 CH_2Cl_2:Et_2O, 5mLx4, Et_2O, 5 mL, 10 mL) afforded **1f** (0.169 g, 97%) as a yellow, free-flowing, powder.

MP: 82°C (decomposition)
IR: 3096, 2937, 2863, 2114, 1758, 1704, 1609, 1586, 1547, 1494, 1421, 1370, 1273, 1197, 1152, 1109, 1013, 892, 853.
HRMS-(ESI)$^+$ (m/z) Found (M–BF_4): 866.0985, Calc'd $C_{39}H_{31}O_{10}N_3F_2I$ requires 866.1017.

(1-diazo-2-oxo-2-(prop-2-yn-1-yloxy)ethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate. (1g)



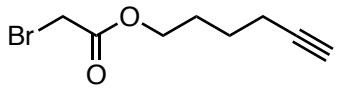
1g

1g was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.537 g, 1.5 mmol), boron trifluoride diethyl etherate (0.24 mL, 2.0 mmol), and propargyl diazoacetate (0.244 g, 2.0 mmol), with a reaction time of 30 minutes. The reaction solution was then transferred to a flask containing a solution of 4:26:70 CH_2Cl_2:40-60 petroleum ether:Et_2O (40 mL), and the resulting mixture was triturated using a sonicator, affording **1g** (0.516 g, 77%) as a yellow, free flowing, powder. **1g** shows good stability (>12 months) when stored in a –20 °C freezer.

MP: 79°C (decomposition)
^{1}H-NMR (500 MHz, CD_3CN) δ: 8.25 (app ddd, *J* = 5.8, 6.6, 8.4 Hz, 1H), 7.40 (app td, *J* = 2.8, 8.8 Hz, 1H), 7.28-7.22 (m, 1H), 4.88 (d, *J* = 2.5 Hz, 2H), 2.93 (t, *J* = 2.5 Hz, 1H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 167.0 (dd, *J* = 11.2, 257 Hz), 161.3 (dd, *J* = 13.6, 256 Hz), 160.0, 139.0 (d, *J* = 10.8), 115.4 (d, *J* = 23.0 Hz), 106.2 (t, *J* = 26.9 Hz), 96.6 (*J* = 23.7 Hz), 76.6, 76.5, 55.0. 40.9 (br).
^{19}F-NMR (376, CD_3CN) δ: –93.7 (d, *J* = 12 Hz), –99.9 (app d, *J* = 12 Hz), -151.7– -151.9 (m, br).

IR: 3292, 2116, 1712, 1695, 1586, 1471, 1424, 1378, 1254, 1184, 1108, 1042, 1003, 962, 864, 814, 765, 731, 673.
HRMS-(ESI)$^+$ (m/z) Found (M–BF_4): 362.9433, Calc'd $C_{11}H_6O_2N_2F_2I$ requires 362.9437.

hex-5-yn-1-yl 2-bromoacetate (S-7)

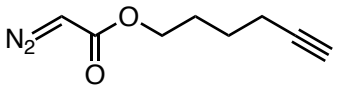


S-7

S-7 was prepared according to General Procedure A using hex–5–yne–1–ol (1.1 mL, 10 mmol), bromoacetyl bromide (2.6 mL, 30 mmol), and sodium bicarbonate (4.29 g, 51 mmol), with a reaction time of 1.5 hours. Diethyl ether was used in the workup. **S-7** was isolated (1.916 g, 87% yield) as a faint brown oil that was of sufficient purity (95%) to be used directly in subsequent transformations.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.21 (t, *J* = 6.4, 2H), 3.84 (s, 2H), 2.26 (td, *J* = 7.0, 2.6 Hz, 2H), 1.97 (t, *J* = 2.6 Hz, 1H), 1.86-1.77 (m, 2H), 1.67-1.57 (m, 2H).
^{13}C-NMR (100 MHz, $CDCl_3$): 167.3, 83.7, 68.0, 65.8, 27.4, 25.9, 24.7 18.0
IR: 3294, 2956, 1732, 1455, 1426, 1278, 1162, 1110, 1019, 986, 958, 926, 889.
HRMS-(ESI)$^+$ (m/z) Found (M+H): 219.0023, Calc'd $C_8H_{12}O_2Br$ requires 219.0021.

hex-5-yn-1-yl 2-diazoacetate (S-8)

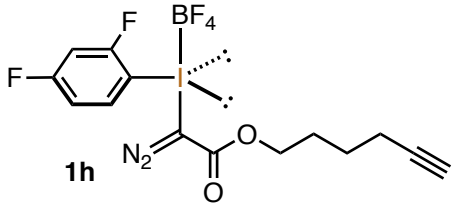


S-8

S-8 was prepared according to General Procedure B using **S-7** (0.78 g, 3.6 mmol), *N,N'*-ditosylhydrazine (1.8 g, 5.3 mmol), and 1,8-diazo-bicyclo-[5.4.0]-undec-7-ene (2.1 mL, 14 mmol), with a reaction time of 1 hour. Diethyl ether was used in the workup. Column chromatography (10% diethyl ether/40-60 petroleum ether) afforded **S-8** (403 mg, 69%) as a yellow oil.

R_f: 0.5 (20% Et_2O:40-60 Petroleum ether)
^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.74 (s, br, 1H), 4.19 (t, *J* = 6.4 Hz, 2H), 2.24 (td, *J* = 7.0, 2.6 Hz, 2H), 1.97 (t, *J* = 2.6 Hz, 1H), 1.83-1.74 (m, 2H), 1.65-1.56 (m, 2H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 166.9 (br), 83.8, 68.7, 64.3, 46.1 (br), 27.8, 25.8, 18.0.
IR: 3292, 2953, 2106, 1681, 1396, 1354, 1240, 1178, 1036, 739, 633.
HRMS-(ESI)$^+$ (m/z) Found (M+H): 167.0813, Calc'd $C_8H_{11}O_2N_2$ requires 167.0815

(1-diazo-2-(hex-5-yn-1-yloxy)-2-oxoethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate (1h)

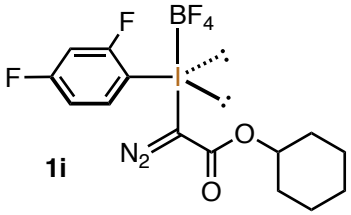


1h

1h was prepared according to General Procedure C using (2,4-difluorophenyl)- λ^3-iodanediyl diacetate (0.398 g, 1.11 mmol), boron trifluoride diethyl etherate (0.20 mL, 1.4 mmol), and **S-8** (0.239 g, 1.44 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (1:9 40-60 petroleum ether:Et_2O, 20 mL x2, 1:3 40-60 petroleum ether:Et_2O, 20 mL) afforded an orange residue. The residue was transferred to a storage vial, and was sonicated with an additional solution of 1:1 40-60 petroleum ether:Et_2O (5 mL) to yield **1h** (0.420 g, 77%) as a viscous, orange oil.

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.21 (ddd, *J* =5.7, 6.2, 9.2 Hz, 1H), 7.37 (app td, *J* = 2.8, 8.9 Hz, 1H), 7.26-7.20 (m, 1H), 4.26, (t, *J* = 6.3 Hz, 2H), 2.24-2.17 (m, 3H), 1.78-1.72 (m, 2H), 1.57-1.50 (m, 2H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 167.0 (dd, *J* = 12, 258 Hz), 161.4 (dd, *J* = 13, 250 Hz), 160.5, 139.0 (app d, *J* = 10 Hz), 115.6 (app d, *J* =23 Hz), 106.3 (app t, *J* = 26 Hz), 96.7 (app d, *J* = 23 Hz), 83.9, 69.1, 67.7, 41.3 (br), 27.3, 24.5, 17.4.
^{19}F-NMR (376 MHz, CD_3CN) δ: –93.9 (d, *J* = 12Hz), –100 (d, *J* = 12 Hz), –151.4 (s), –151.5 (s).
IR: 3289, 2920, 2111, 1705, 1586, 1471, 1425, 1270, 1193, 1152, 1010, 964, 851, 729.
HRMS (ESI) (m/z) Found (M–BF_4): 404.9907, Calc'd $C_{14}H_{12}F_2O_2N_2I$ requires 404.9906.

(2-(cyclohexyloxy)-1-diazo-2-oxoethyl)(2,4-difluorophenyl)iodonium tetrafluoroborate (1i)



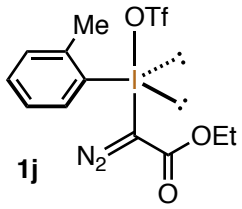
1i

1i was prepared according to General Procedure C using (2,4-difluorophenyl)-λ^3-iodanediyl diacetate (0.361 g, 1.0 mmol), boron trifluoride diethyl etherate (0.18 mL, 1.3 mmol), and cyclohexyl diazoacetate (0.214 g, 1.27 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (9:1 diethyl ether:40-60 petroleum ether, 20 mL, followed by 1:1 diethyl ether:40-60 petroleum ether, 20 mL) afforded **1i** (1.00 g, 87%) as a yellow, free flowing, powder. Note: additional precipitate can be collected. While the carbon possessing the diazo moiety was not observable by ^{13}C-NMR, the IR stretch[8] at 2114 cm^{-1} as well as the correct mass being observed confirms the presence of the diazo group.

MP: 87 °C (decomp)

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.21 (app ddd, *J* = 5.8, 6.8, 9.2 Hz, 1H), 7.37 (app dt, *J* = 2.8, 8.9 Hz, 1H), 7.26-7.20 (m, 1H), 4.86 (m, 1H), 1.87-1.79 (m, 2H), 1.75-1.66 (m, 2H), 1.57-1.45 (m, 3H), 1.44-1.26 (m, 3 H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.0 (app dd, *J* = 11.7, 257 Hz), 161.4 (app dd, *J* = 13.7, 257 Hz), 15.9.8, 139.0 (d, *J* =11.0 Hz), 115.5 (app dd, *J* = 2.74, 23.7 Hz), 106.3 (app t, *J* = 11.9 Hz), 96.7 (app dd, *J* = 3.4, 23.4 Hz), 77.4, 31.0 (2C), 24.8, 23.1 (2C).
^{19}F-NMR (376 MHz, CD_3CN) δ: –92.8 (d, *J* = 12Hz), –100 (d, *J* = 12 Hz), –151.9–-152.0 (m).
IR: 2946, 2865, 2114, 1698, 1687, 1586, 1471, 1423, 1270, 1192, 1114, 1033, 964, 730.
HRMS-$(ESI)^+$ (m/z) Found (M-BF_4): 407.0065, Calc'd $C_{14}H_{14}F_2IN_2O_2$ requires 407.0063.

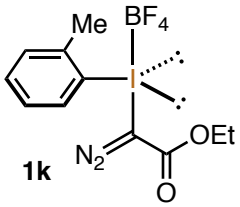
(1-diazo-2-ethoxy-2-oxoethyl)(*o*-tolyl)iodonium trifluoromethanesulfonate (1j)



1j

1j was prepared according to General Procedure C using (*o*-tolyl)-λ^3-iodanediyl diacetate (0.503 g, 1.5 mmol), trimethylsilyl trifluoromethansolfuonate (0.36 mL, 3.2 mmol), and ethyl diazoacetate (0.39 mL, 3.2 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (5:20:75 CH_2Cl_2:40-60 petroleum ether:Et_2O, 20 mL, 1:1 Et_2O:40-60 petroleum ether, 20 mL) afforded an orange residue. The residue was transferred to a storage vial, and was sonicated with an additional solution of Et_2O:40-60 petroleum ether (10 mL) and 40-60 petroleum ether (10 mL) to yield **1j** (0.258 g, 37%) as an orange, sticky oil.
^{1}H-NMR (500 MHz, CD_3CN) δ: 8.19 (dd, *J* = 1.1, 8.3 Hz, 1H), 7.66 (dt, *J* = 1.1, 7.5 Hz, 1H), 7.61-7.58 (m, 1H), 7.34 (tdd, 0.5, 2.0, 7.0 Hz, 1H), 4.27 (q, 7.0 Hz, 2H), 2.90 (s, 3H), 1.25 (t, *J* = 7.0 Hz), 3H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 161.2, 141.8, 137.6, 134.1, 132.0, 129.6, 120.8 (q, *J* =320 Hz), 64.1, 39.7 (br), 24.8, 13.5.
^{19}F-NMR (376 MHz, CD_3CN) δ: –79.7.
IR: 3093, 3054, 3018, 2998, 2970, 2108, 1704, 1464, 1448, 1369, 1265, 1235, 1224, 1164, 1024, 759, 732.
HRMS-$(ESI)^+$ (m/z) Found (M–OTf): 330.9944, Calc'd $C_{11}H_{12}O_2N_2I$ requires 330.9938

(1-diazo-2-ethoxy-2-oxoethyl)(*o*-tolyl)iodonium tetrafluoroborate (1k)



1k

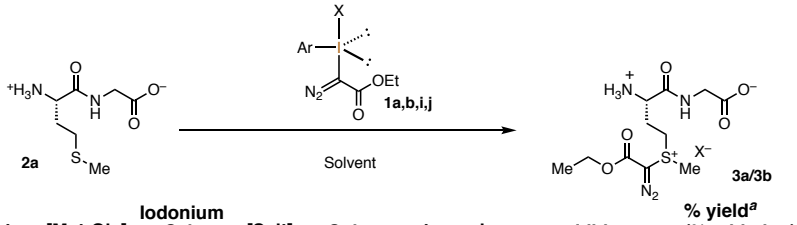
1k was prepared according to General Procedure C using (*o*-tolyl)-λ^3-iodanediyl diacetate (0.505 g, 1.5 mmol), boron trifluoride diethyl etherate (0.24 mL, 2.0 mmol), and ethyl diazoacetate (0.24 mL, 2.0 mmol), with a reaction time of 30 minutes. Trituration using a sonicator (5:20:75 CH_2Cl_2:40-60 petroleum ether:Et_2O, 20 mL, 40-60 petroleum ether, 10 mL) afforded an orange residue. The residue was transferred to a storage vial, and was sonicated with an additional solution of 5:20:75 CH_2Cl_2:40-60 petroleum ether:Et_2O (10 mL) and 40-60 petroleum ether (10 mL) to yield **1k** (0.662 g, 99%) as an orange, sticky oil of ca 90% purity. While the carbon possessing the diazo moiety was not observable by ^{13}C-NMR, the IR stretch[8] at 2114 cm^{-1} as well as the correct mass being observed confirms the presence of the diazo group.

^{1}H-NMR (500 MHz, CD_3CN) δ: 8.21 (dd, *J* = 0.9, 8.2 Hz, 1H), 7.67 (app td, *J* = 0.9, 7.4 Hz, 1H), 7.67-7.61 (m, 1H), 7.41-7.35 (m, 1H), 4.30 (q, *J* = 7.2 Hz, 2H), 2.71 (s, 3H), 1.28 (t, *J* = 7.2 Hz, 3H).
^{13}C-NMR (125 MHz, $CDCl_3$) δ: 160.7, 141.9, 137.7, 134.3, 132.0, 129.7, 121.0, 64.2, 24.7, 13.4.
^{19}F-NMR (376 MHz, CD_3CN) δ: -151.8– -152 (m, br).
IR: 2114, 1698, 1465, 1271, 964 (br), 730.
HRMS-$(ESI)^+$ (m/z) Found ($M–BF_4$): 330.9944, Calc'd $C_{11}H_{12}O_2N_2I$ requires 330.9938.

Initial reaction optimization on model peptides

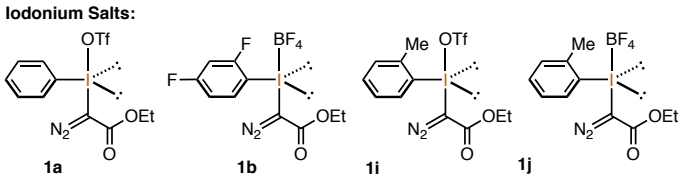
A general procedure used for the optimization of the functionalization of **2a** is as follows: To a stirring solution of **2a** was added a solution containing the desired additive (if any was used), followed by a solution containing the relevant iodonium salt. The resulting mixture was allowed to stir for one hour. The resulting solution was then analyzed directly *via* ^{1}H-NMR using dimethylformamide as an internal standard.

Table S1: Optimization of the labeling of **2a** with hypervalent iodine reagents.

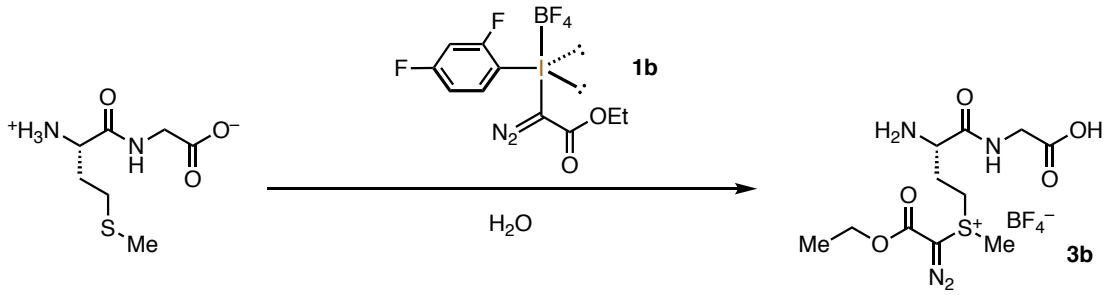


Entry	[Met-Gly]	Iodonium Salt	[Salt]	Solvent	atmosphere	additive	% yield[a] (%oxidation)
1	50 mM	1a	50 mM	H_2O	ambient	——	28% (N/A)
2	50 mM	1i	50 mM	H_2O	ambient	——	38% (N/A)
3	50 mM	1i	100 mM	H_2O	ambient	——	76% (N/A)
4	50 mM	1i	150 mM	H_2O	ambient	——	85% (N/A)
5	50 mM	1j	150 mM	D_2O	ambient	——	95% (N/A)
6	5 mM	1j	100 mM	D_2O	ambient	——	68% (N/A)
7	5 mM	1b	50 mM	D_2O	ambient	——	72% (17%)
8	5 mM	1b	50 mM	D_2O	sparged w/ N_2 30 min	——	79% (<5%)
9	5 mM	1b	50 mM	D_2O	ambient	TEMPO (10 mM)	70% (<5%)
10	5 mM	1b	50 mM	D_2O	ambient	Ascorbic Acid (10 mM)	73% (<5%)
11	5 mM	1b	50 mM	D_2O	ambient	thiourea (10 mM)	94% (<5%)
12	500 μM	1b	50 mM	D_2O	ambient	thiourea (10 mM)	>90% (N/A)
13	500 μM	1b	50 mM	D_2O	ambient	——	trace (N/A)
14	500 μM	1b	50 mM	D_2O	sparged w/ N_2 30 min	——	~50% (N/A)
15	5 mM	1b	25 mM	D_2O	ambient	thiourea (10 mM)	81% (<5%)
16	5 mM	1b	50 mM	D_2O	ambient	thiourea (5 mM)	74% (9%)
17	5 mM	1b	50 mM	D_2O	ambient	thiourea (20 mM)	>95% (trace)
18	5 mM	1b	50 mM	D_2O	ambient	thiourea (50 mM)	34% (trace)

[a]reactions were stirred for one hour, and were analzyed directly via ^{1}H–NMR using DMF (13 mM) as an internal standard.



((*S*)-3-amino-4-((carboxymethyl)amino)-4-oxobutyl)(1-diazo-2-ethoxy-2-oxoethyl)(methyl)sulfonium tetrafluoroborate (3b)



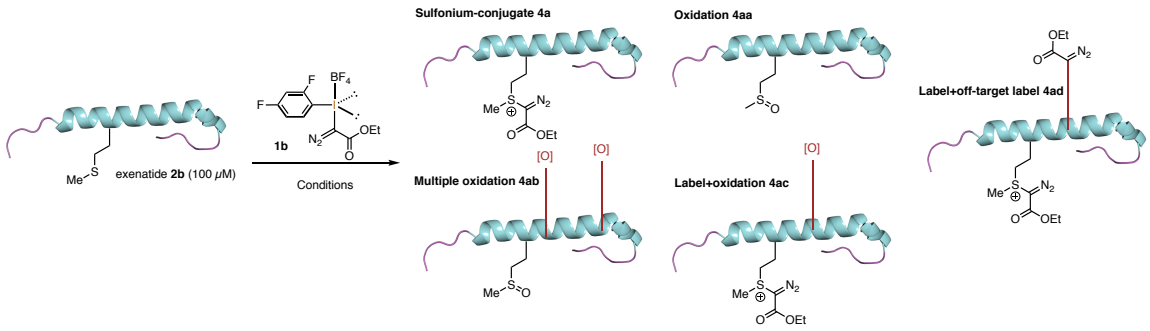
A round-bottomed flask equipped with a magnetic stir-bar was charged with methionine-glycine (11 mg, 0.052 mmol) and then evacuated and refilled with N_2. To the flask was added a freshly degassed solution of **1b** (0.3 M in H_2O, 0.5 mL), and the resulting solution was allowed to stir for 1 hour at room temperature. The resulting heterogeneous solution was then diluted with water and ethyl acetate and the layers separated by decanting the organic layer. The aqueous layer was washed twice with ethyl acetate in the same fashion. The aqueous layer was then evaporated to dryness *in vacuo*. The resulting faint yellow residue was then redissolved in a minimal amount of acetonitrile and precipitated with ether in a 4°C refrigerator. The supernatant was decanted, and the residue was dried *in vacuo* to yield **3b** as a faint yellow residue (17 mg, 82%, 1:1 mixture of diastereomers). **3b** is considerably hygroscopic and should be stored in a –20°C freezer.

^{1}H-NMR (500 MHz, D_2O) δ: 4.42 (app q, *J* = 7.0 Hz, 2H), 4.24 (app t, *J* = 6.2 Hz, 1H), 4.05-3.90 (m, 3H), 3.81-3.70 (m, 1H), 3.34 (s, 1.5H), 3.33 (s, 1.5H), 2.57-2.39 (m, 2H), 1.34 (app t, *J* = 7.0 Hz, 3H).
^{13}C-NMR (125 MHz, D_2O) δ: 174.2 (br, 2C), 167.8, 167.7, 161.1 (2C), 64.5, 53.7 (br), 51.3, 51.2, 42.2 (2C), 39.4, 39.2, 23.4 (br, 2C), 25.5, 25.4, 13.2.
IR: 2989, 2150, 1690 (br), 1547 (br), 1393, 1370, 1277, 1003 (br), 737.
HRMS-(ESI)$^+$ (m/z) Found (M^+): 319.1016, Calc'd $C_{11}H_{19}O_5N_4$ requires 319.1071.

Protein Modification

A general procedure used for the optimization of the functionalization of exenatide•OAc with **1b** is as follows: To a stirring solution of **exenatide•OAc** was added a solution containing the desired additive (if any was used), followed by a solution containing **1b**. The resulting mixture was allowed to stir for the time depicted. The resulting solution was then analyzed directly *via* LC/MS.

Table S2. Optimization of the labeling of Exenatide•OAc with **1b**.



Entry	[1b]	Additive	Solvent	atmosphere	t °C	T(min)	%conversion	%4a	%4aa	%4ab	%4ac	%4ad
1[a]	50 mM	none	H_2O	ambient	RT	30	>95%	Decomposition				
2[a]	50 mM	none	1:1 H_2O: CH_3CN	ambient	RT	30	>95%	10%	22%	—	—	—
3[a]	50 mM	TEMPO (5 mM)	H_2O	ambient	RT	30	>95%	12%	8%	—	—	—
4[a]	50 mM	TEMPO (5 mM)	1:1 H_2O: CH_3CN	ambient	RT	30	>95%	—	—	81%	19%	—
5[a]	50 mM	thiourea (20 mM)	1:1 H_2O: CH_3CN	ambient	RT	5	>95%	68%	2%	—	27%	4%
6[a]	50 mM	thiourea (20 mM) TEMPO (5 mM)	1:1 H_2O: CH_3CN	ambient	RT	5	>95%	93%	1%	—	1%	4%
7[a]	50 mM	thiourea (20 mM) TEMPO (5 mM) Formic Acid (5 mM)	1:1 H_2O: CH_3CN	ambient	RT	5	>95%	>95%	1%	—	1%	1%
8[a]	50 mM	thiourea (20 mM) TEMPO (5 mM) Formic Acid (5 mM)	H_2O	ambient	RT	5	>95%	>95%	1%	—	1%	1%

[a] Reaction Conversions and ratios estimated by LC/MS TIC.

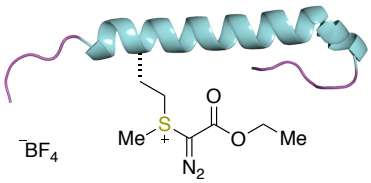
General Optimal Procedure A for Protein Modification

A 2 mL vial equipped with a magnetic stirrer was charged with a solution containing the desired protein (1 mM in 0.1M thiourea (aq), 20 μL). To the vial was added thiourea (100 mM in H_2O, 20μL), formic acid (100 mM, 10 μL), and H_2O (20 μL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (33 mM in H_2O, 30 μL) and the vial was stirred vigorously. To the stirring solution was added the iodonium salt (100 mM in CH_3CN or H_2O, 100 μL) and the resulting solution stirred for 5 minutes at 0° C. The resulting mixture was then extracted twice with diethyl ether or ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS.

General Optimal Procedure B for Protein Modification

A 2 mL vial equipped with a magnetic stirrer was charged with a solution containing the desired protein (1 mM in 100 mM thiourea (aq), 20 μL). To the vial was added thiourea (100 mM in H_2O, 20μL), and H_2O (30 μL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (33 mM in H_2O, 30 μL) and the vial was stirred vigorously. To the stirring solution was added the iodonium salt (100 mM in CH_3CN or H_2O, 100 μL) and the resulting solution stirred for 5 minutes at 0° C. The resulting mixture was then extracted twice with diethyl ether or ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS.

Functionalization of Exenatide acetate with 1b to form 4a.



The labeling of exenatide was performed at 0°C using general procedure A using **1b** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min) and was judged to have proceeded in >95% conversion (**Figure S1-A**).

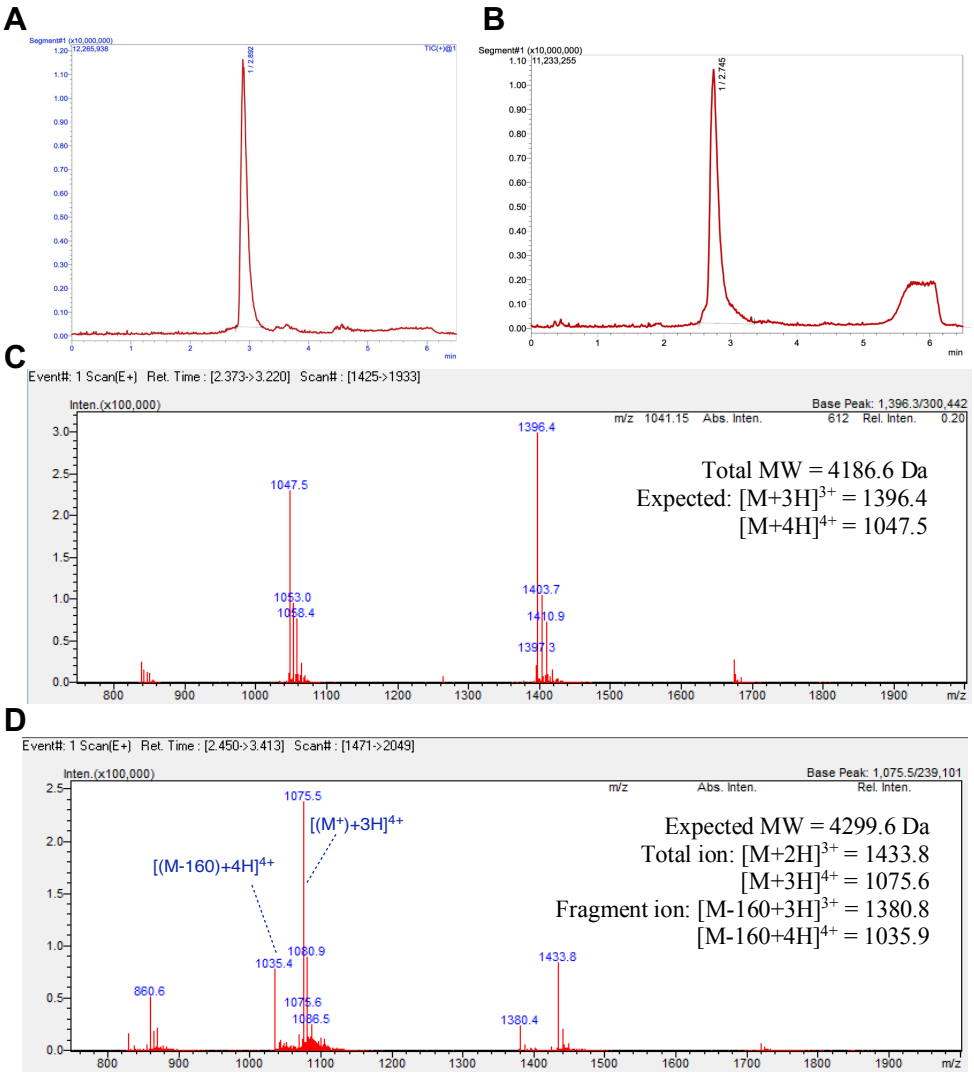
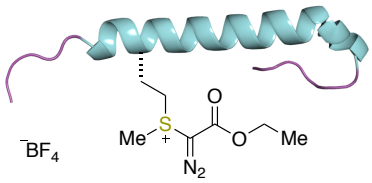


Figure S1-A. **(A)** Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1b**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4b** (positive ionization mode, TIC from T_r=2.31-3.16 min.)

Functionalization of Exenatide acetate with 1b to form 4a in pure aqueous conditions.



The labeling of exenatide was performed at room temperature using general procedure A using **1b** (100 mM in H_2O) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min) and was judged to have proceeded in >95% conversion (**Figure S1-B**).

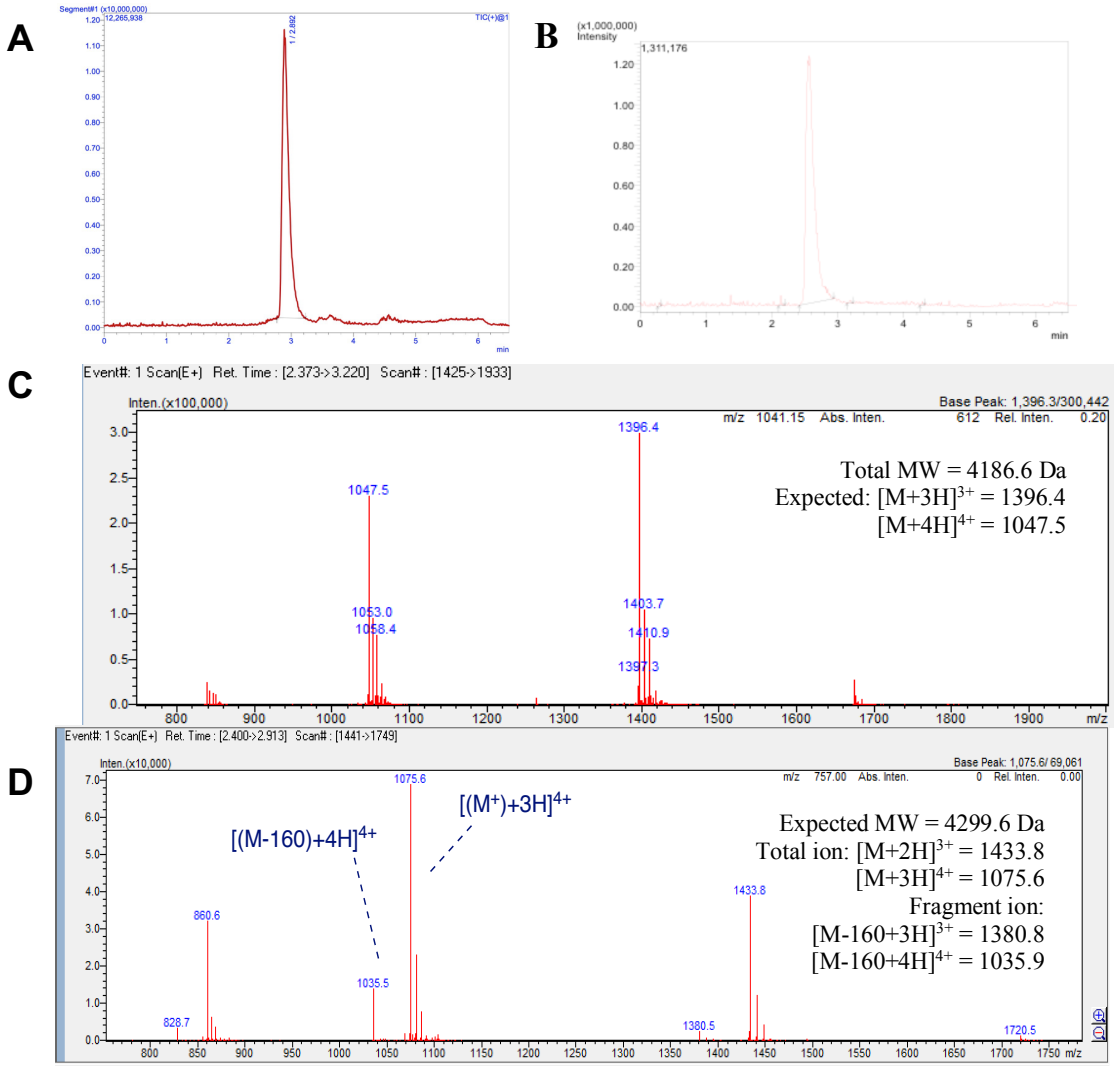


Figure S1-B. **(A)** Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1b**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4a** (positive ionization mode, TIC from T_r=2.40-2.91 min.)

Functionalization of Exenatide acetate with 1b to form 4a using a pre-made mixture (stock solution) of reagents.

The labeling of exenatide was performed as follows: to a solution of exenatide (1 mM, 20 μL, H_2O) was added an aqueous stock solution (80 μL) containing thiourea (50 mM), TEMPO (12.5 mM), and formic acid (12.5 mM). The resulting solution was chilled and rapidly stirred. To the rapidly stirring solution was added **1b** (100 μL, CH_3CN), and the resulting stirred for 5 minutes. and the resulting solution stirred for 5 minutes at 0° C. The resulting mixture was then extracted twice with diethyl ether. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS, Pentafluorophenyl column (50 x 2.1 mm, 2.6μm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min) and was judged to have proceeded in >95% conversion (**Figure S1-C**).

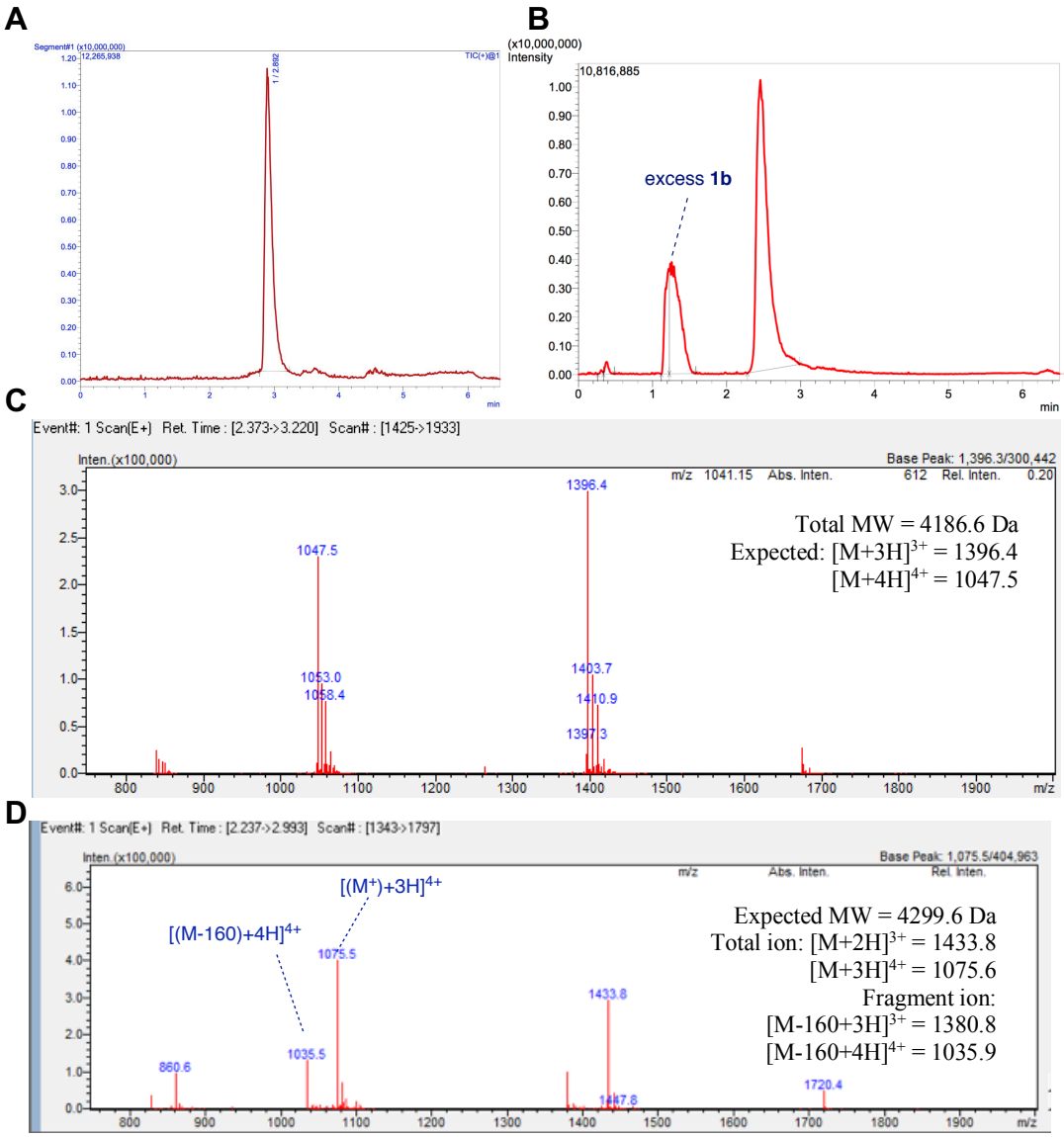
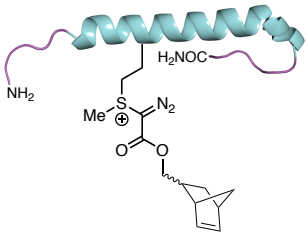


Figure S1-C. (A) Mass trace of parent exenatide•OAc. (B) Mass trace of the crude mixture between exenatide and 1b. (C) Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) (D) Mass chromatogram of the crude reaction to form 4b (positive ionization mode, TIC from T_r=2.29-3.69 min.)

Functionalization of Exenatide•OAc with 1d to form 4g.



The labeling of exenatide was performed according to general procedure A using **1d** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S2**).

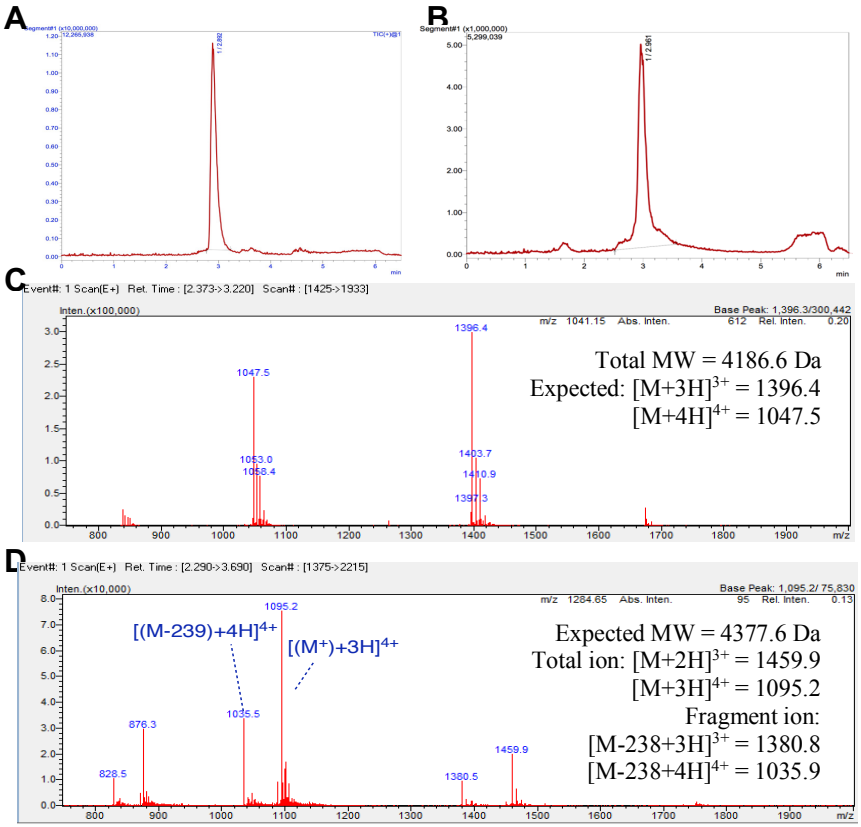
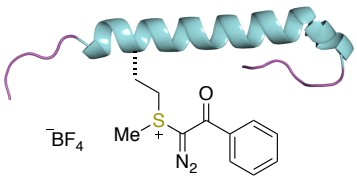


Figure S2. (**A**) Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1d**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4b** (positive ionization mode, TIC from T_r=2.29-3.69 min.)

Functionalization of Exenatide•OAc with 1e to form 4c



A 2 mL vial equipped with a magnetic stirrer was charged with a solution containing Exenatide•OAc (1 mM in H_2O, 40 µL). To the vial was added *N*-phenylthiourea (0.1 M in H_2O, 40µL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (200 mM in CH_3CN, 30 µL) and the vial was stirred vigorously. To the stirring solution was added the iodonium salt (0.1 M in CH_3CN, 100 µL) and the resulting solution stirred for 30 minutes at 0° C. The resulting mixture was then extracted twice with diethyl ether or ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in 87% conversion (**Figure S3**).

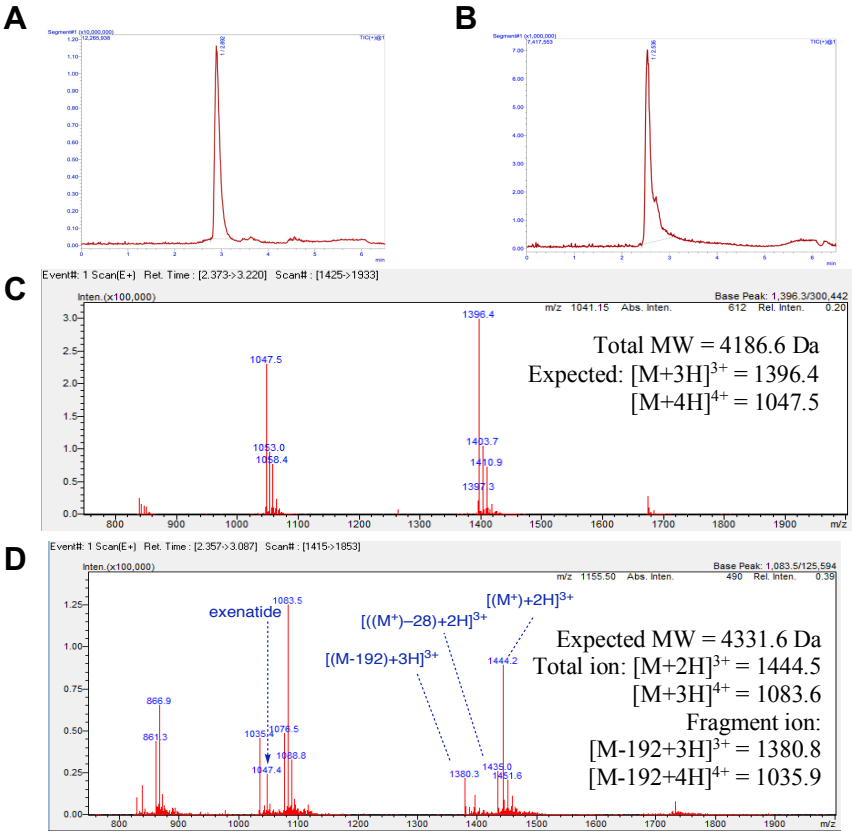
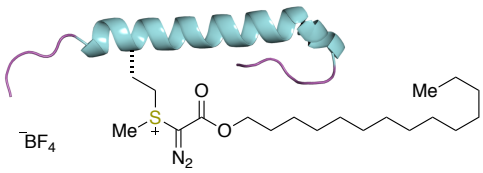


Figure S3. (**A**) Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1e**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4d** (positive ionization mode, TIC from T_r=2.36-3.07 min.)

Functionalization of Exenatide•OAc with 1c to form 4d.



The labeling of exenatide was performed according to general procedure A using **1c** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion with an estimated 94% formation of **4d** and a 2% formation of exenatide sulfoxide (47:1 ratio) (**Figure S4**).

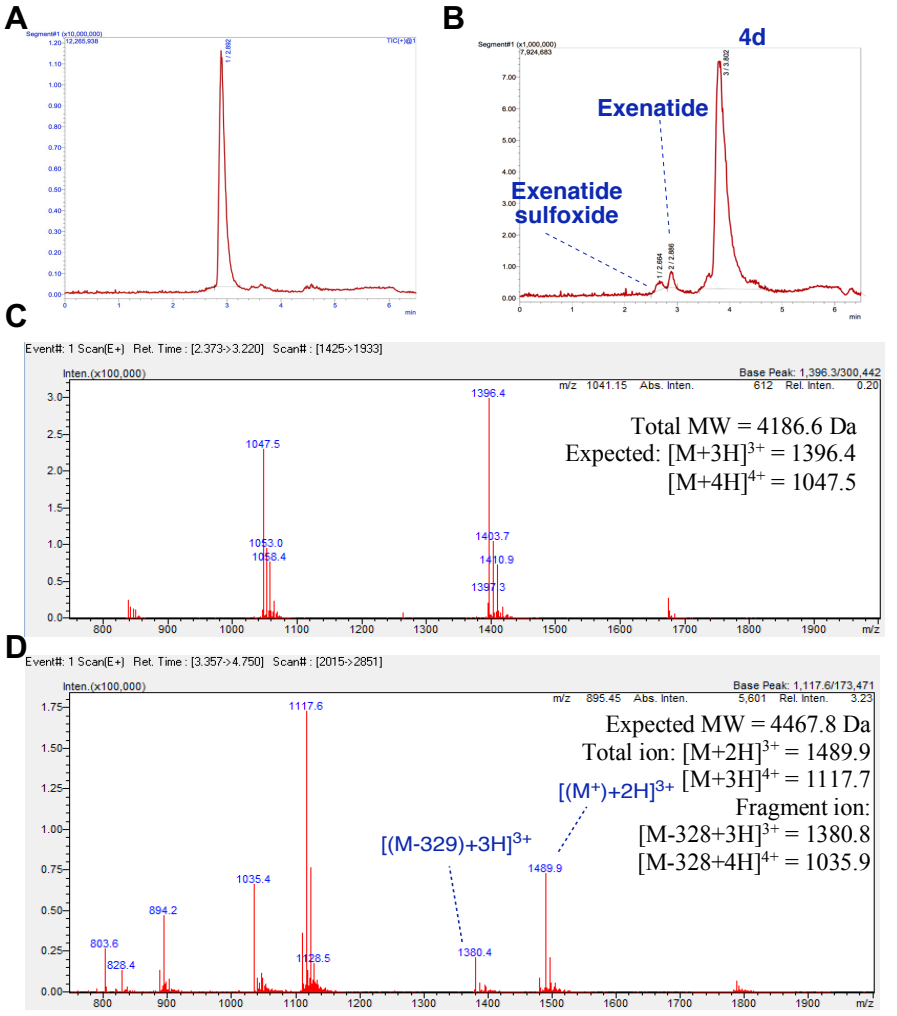
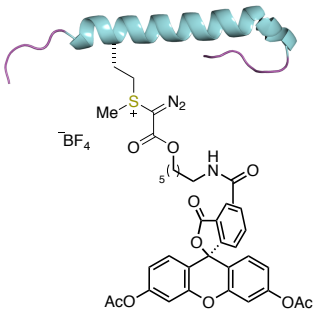


Figure S4. (**A**) Mass trace of parent exenatide•OAc. (**B**) Mass trace of the crude mixture between exenatide and **1c**. (**C**) Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) (**D**) Mass chromatogram of the crude reaction to form **4d** (positive ionization mode, TIC from T_r=3.36-4.75 min.)

Functionalization of Exenatide•OAc with 1f to form 4e.



The labeling of exenatide was performed according to general procedure A using **1f** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/ MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in 84% conversion, with an estimated 80% formation of **4e** and 4% exenatide sulfoxide (20:1 ratio) (**Figure S5**).

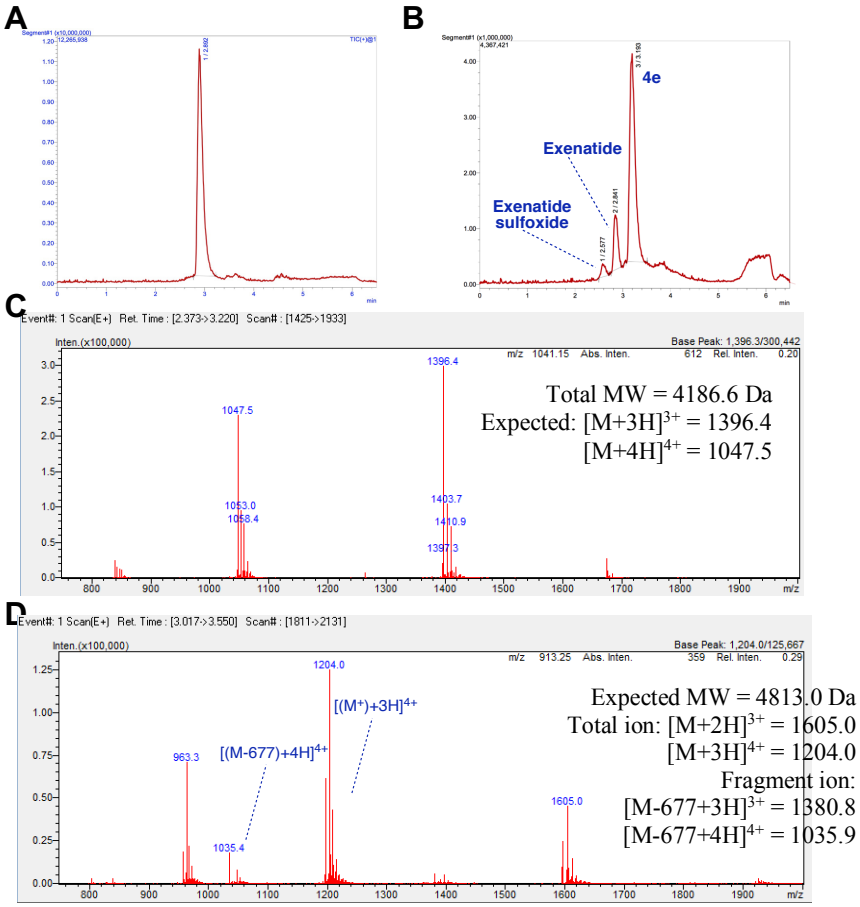
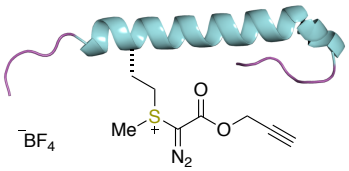


Figure S5. (**A**) Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1f**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4e** (positive ionization mode, TIC from T_r=3.02-3.55 min.)

Functionalization of Exenatide•OAc with 1g to form 4f



The labeling of exenatide was performed according to general procedure A using **1g** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/ MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S6**).

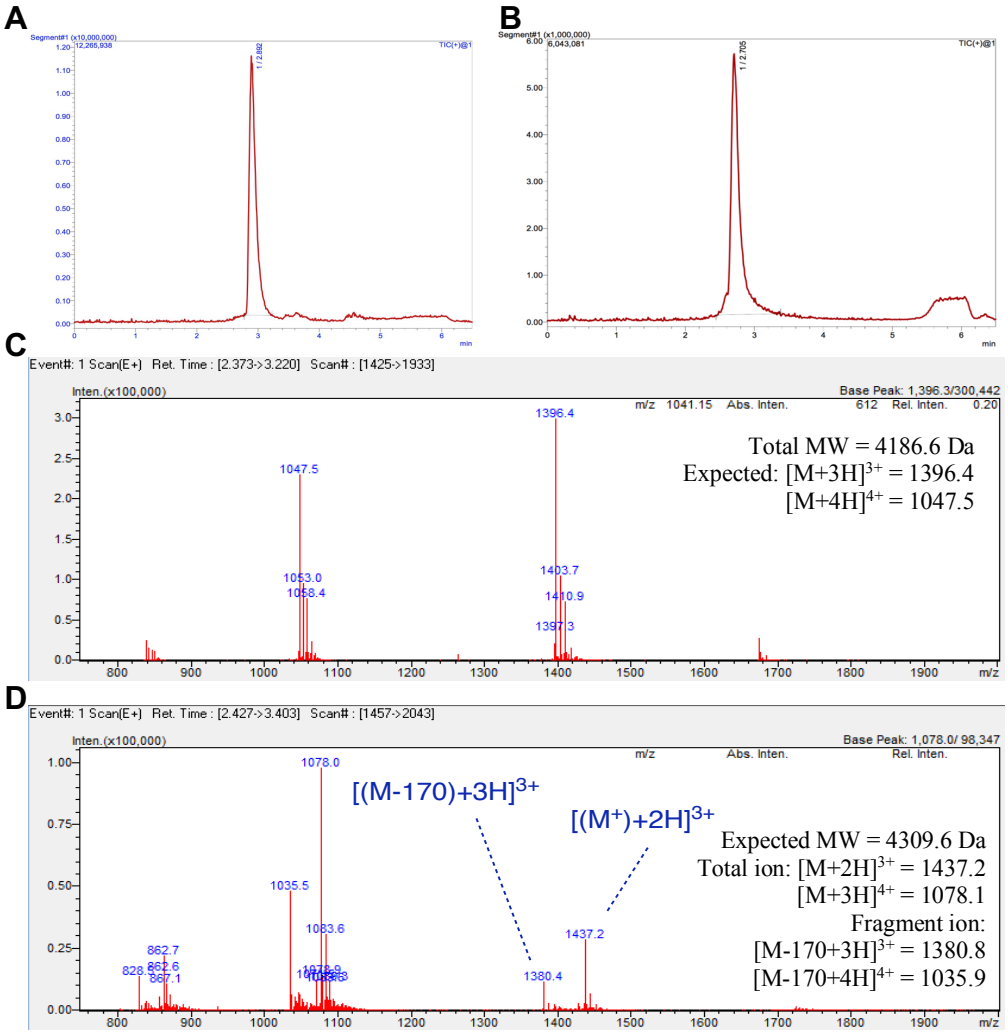


Figure S6. (**A**) Mass trace of parent exenatide•OAc. **(B)** Mass trace of the crude mixture between exenatide and **1g**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4f** (positive ionization mode, TIC from T_r=2.43-3.40 min.)

Large Scale Synthesis of 4f

A 2 mL vial equipped with a magnetic stirrer was charged with Exenatide•OAc (2.8 mM in 100 mM thiourea (aq), 70 µL (~0.83 mg peptide)). To the vial was added formic acid (100 mM, 20 µL), and H_2O (30 µL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (33 mM in H_2O, 50 µL) and the vial was stirred vigorously. To the stirring solution was added **1g** (100 mM in CH_3CN 180 µL) and the resulting solution stirred for 5 minutes at 0° C. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. Purification *via* reverse-phase HPLC* (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95% B over 1 min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **4f** as a sticky, off-white solid (1 mg) that was immediately redissolved in H_2O (500 µL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.4 mg/mL solution (79% isolated yield). *Note: Purification of **4f** should be carried out expediently as prolonged purification times can lead to low levels of peptide backbone hydrolysis.

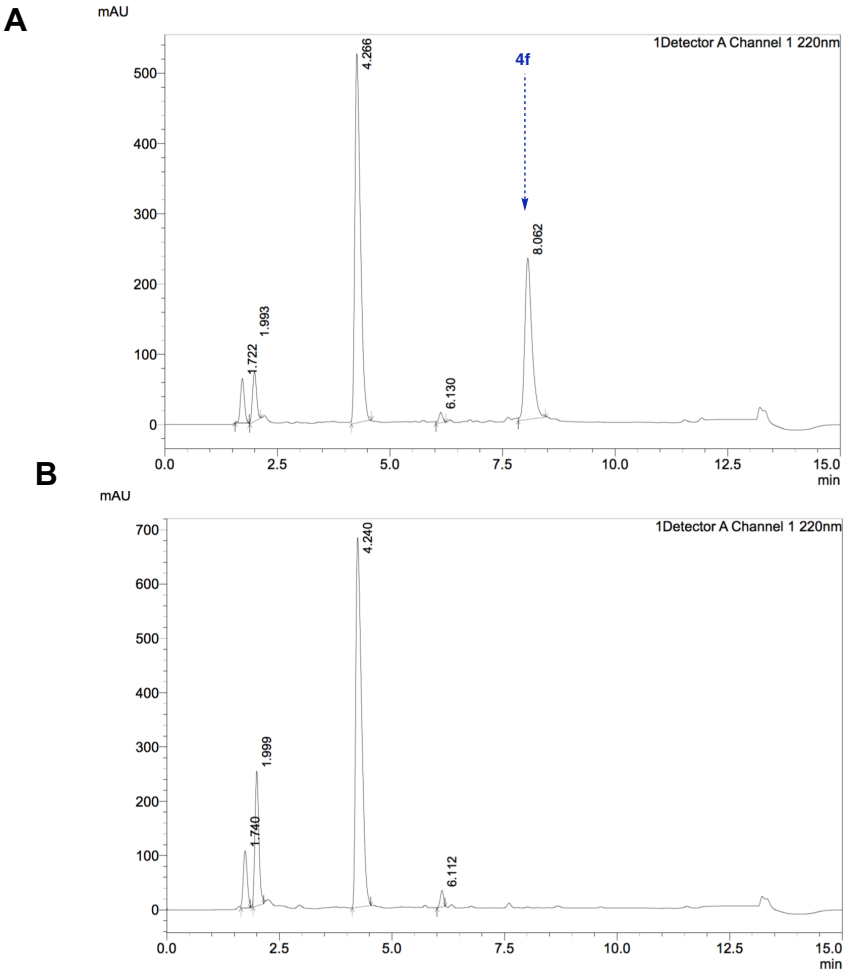


Figure S7. (**A**) HPLC trace of the crude reaction mixture of exenatide•OAc and **1g**. **(B)** HPLC trace of a similar reaction wherein exenatide•OAc was NOT added.

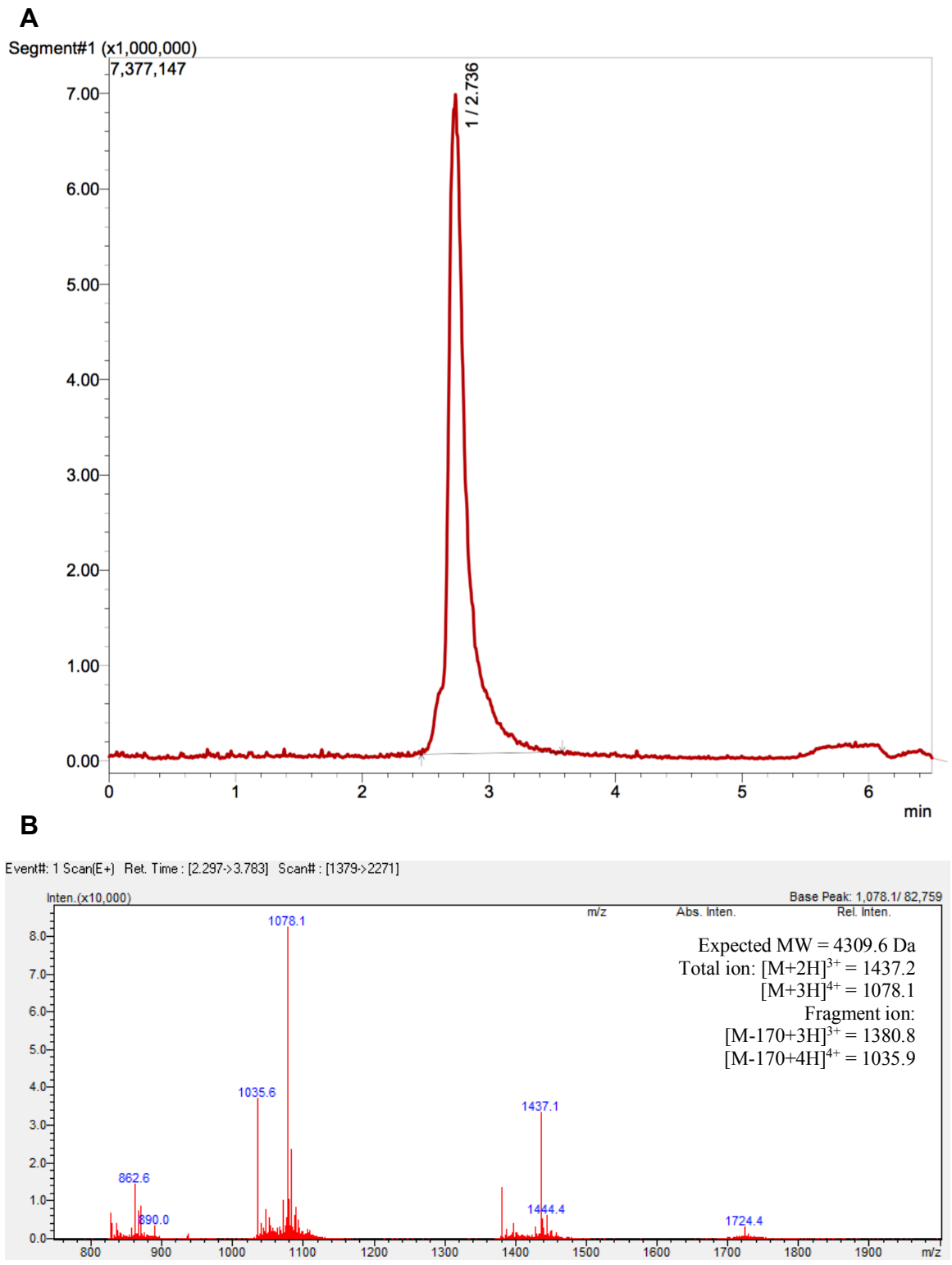


Figure S8. (**A**) Mass trace of purified **4f**. **(B)** Mass spectrum of purified **4f.**

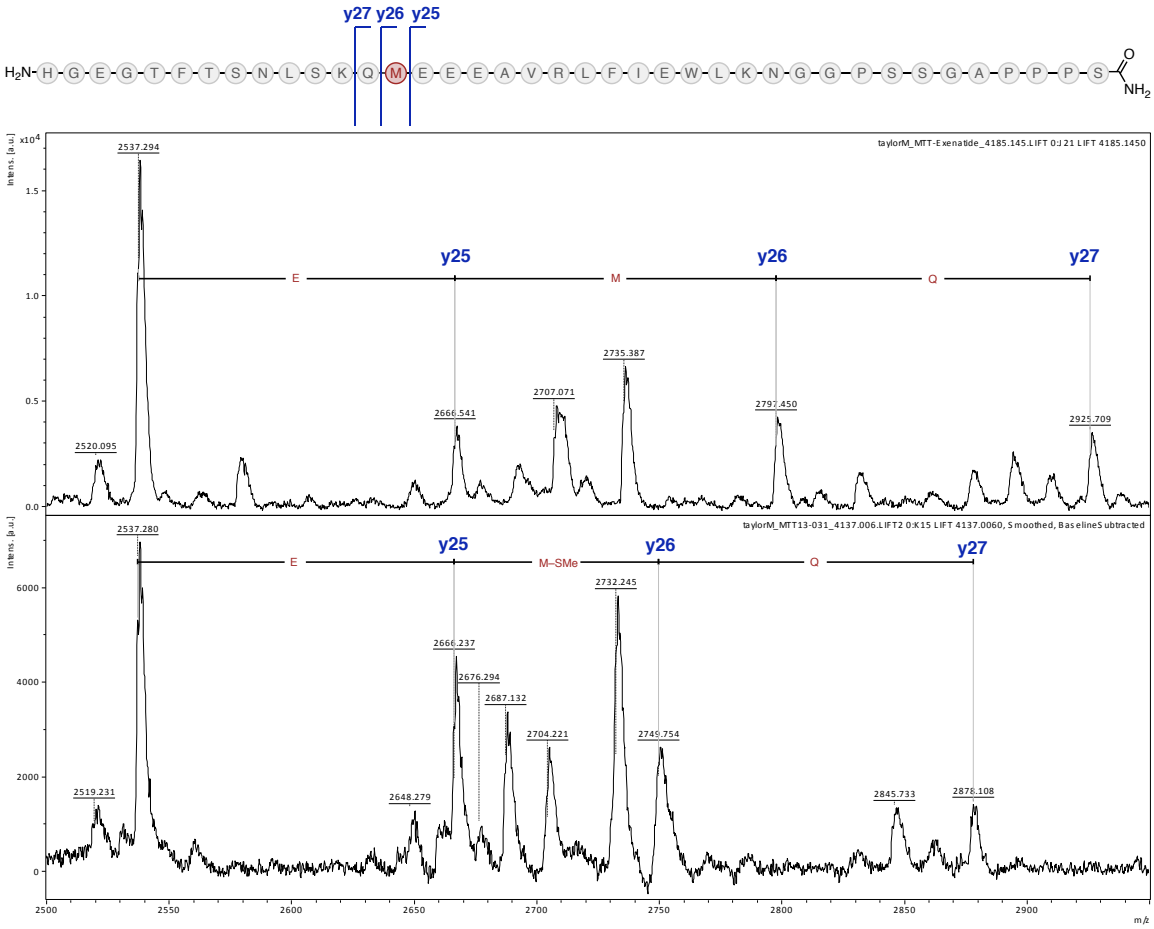


Figure S9. (**A)** MS/MS chromatogram of Exenatide (**4**) highlighting the y25, y26, and y27 fragments. **(B)** MS/MS chromatogram of conjugate **4f** highlighting the y25, y26, and y27 fragments of (**4f**–170)$^+$ fragment. The difference of 48 mass units on the y26 mass between the two spectra confirm modification of the methionine residue in exenatide.

CD spectra of exenatide conjugate 4f

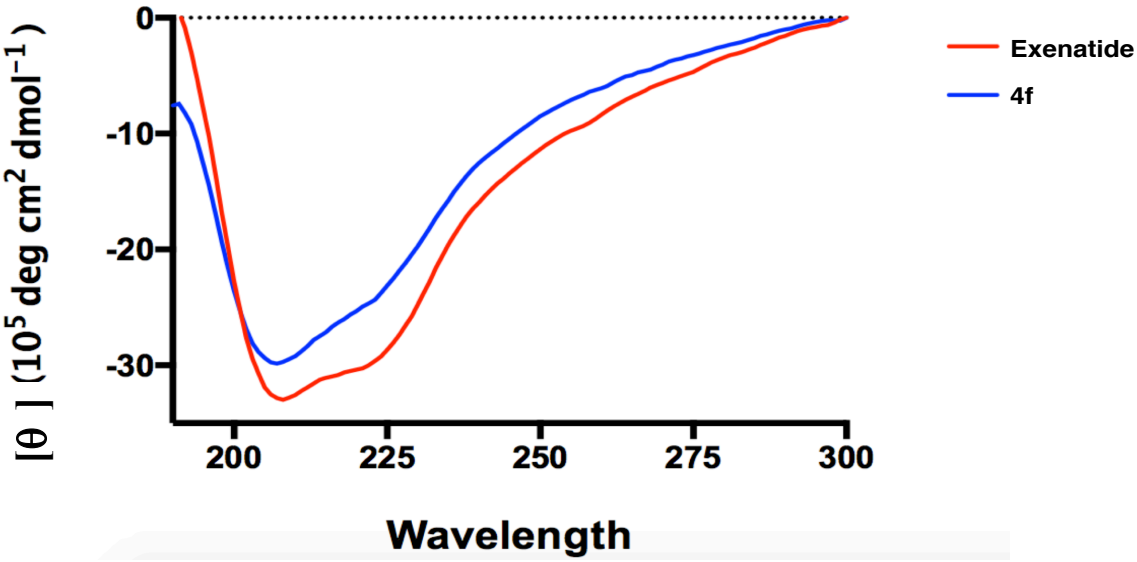
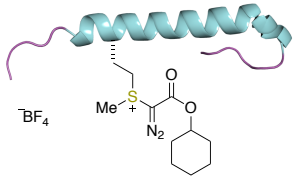


Figure S10. CD spectra of **4f** (5 µM) in 5 mM pH 7.4 phosphate buffer (blue line), and Exenatide (5 µM) in 5 mM pH 7.4 phosphate buffer (red line). **4f** displays the diagnostic profile of a helical peptide and indicates that the secondary structure of the peptide is not significantly affected by the methionine modification.

Functionalization of Exenatide•OAc with 1i to form 4b



A 2 mL vial equipped with a magnetic stirrer was charged with Exenatide•OAc (1.8 mM in 100 mM thiourea (aq), 160 µL (~1.2 mg peptide)). To the vial was added formic acid (100 mM, 40 µL), and H_2O (80 µL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (67 mM in H_2O, 120 µL) and the vial was stirred vigorously. To the stirring solution was added **1i** (100 mM in CH_3CN 400 µL) and the resulting solution stirred for 5 minutes at 0° C. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6μm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S11**).

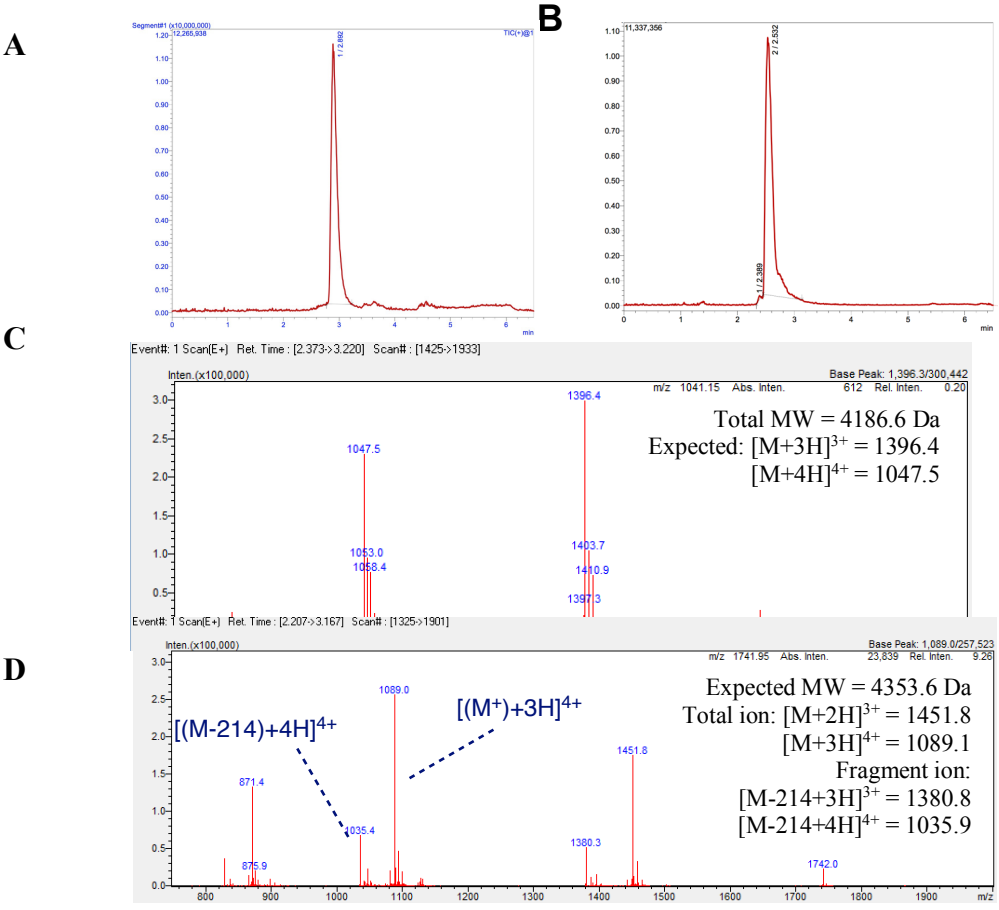
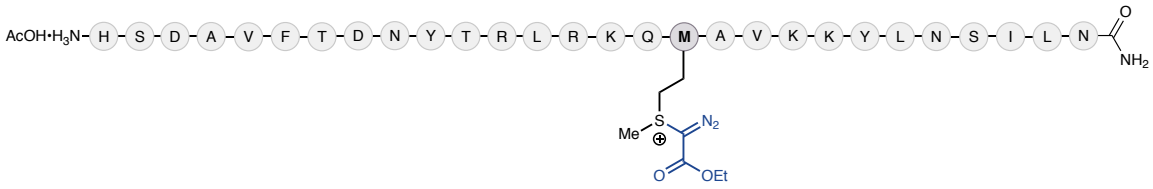


Figure S11. (**A**) Mass trace of parent exenatide•OAc. (**B**) Mass trace of the crude mixture between exenatide and **1i**. (**C**) Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.37-3.22 min.) (**D**) Mass chromatogram of the crude reaction to form **4b** (positive ionization mode, TIC from T_r=2.64-3.65 min.)

Functionalization of Aviptadil•OAc with 1b to form 5.



The labeling of aviptadil was performed according to general procedure B using **1b** (100 mM in H_2O) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S12**).

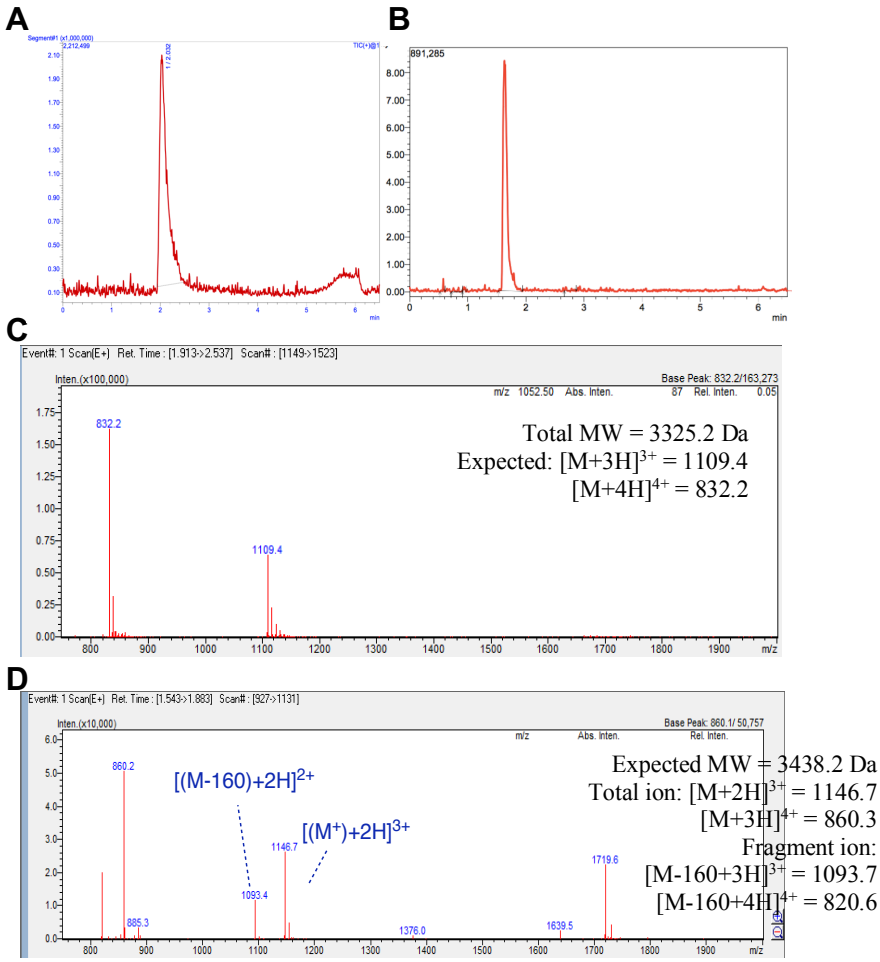


Figure S12. (**A)** Mass trace of parent Aviptadil•OAc. **(B)** Mass trace of the crude mixture between Aviptadil and **1b**. **(C)** Mass chromatogram of Aviptadil•OAc (positive ionization mode, TIC from T_r=1.91-2.54 min.) **(D)** Mass chromatogram of the crude reaction to form **5** (positive ionization mode, TIC from T_r=1.81-2.88 min.)

Large Scale Synthesis of 5

A 2 mL vial equipped with a magnetic stirrer was charged with Aviptadil•OAc (~1.4 mg, ~0.4 µmol). To the vial was added thiourea (100 mM in H_2O, 160 µL), and H_2O (120 µL). To the solution was added TEMPO (67 mM in H_2O, 120 µL) and the vial was stirred vigorously. To the stirring solution was added **1b** (100 mM in H_2O 400 µL) and the resulting solution stirred for 5 minutes at room temperature. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. Purification *via* reverse-phase HPLC* (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95% B over 1 min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **5** as a sticky, off-white solid that was immediately redissolved in H_2O (500 µL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.3 mg/mL solution (>90% isolated yield). *Note: Purification of **5** should be carried out expediently as prolonged purification times can lead to low levels of peptide backbone hydrolysis.

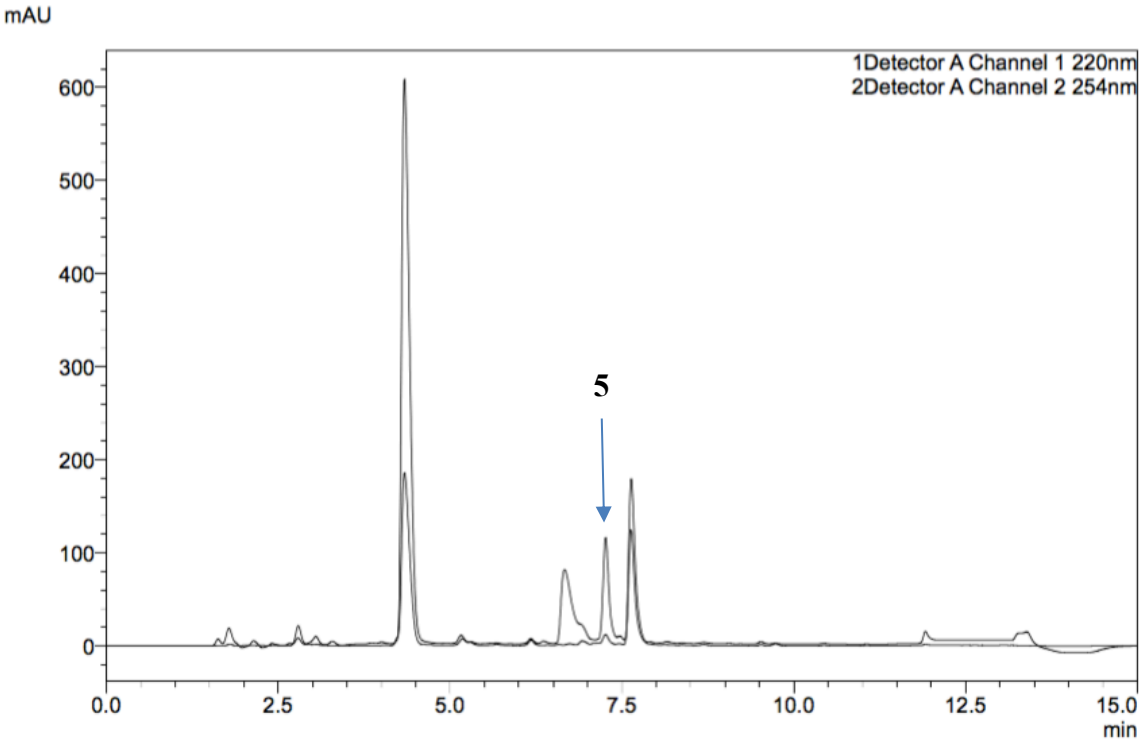


Figure S13. (**A)** HPLC trace of the crude reaction mixture of aviptadil•OAc and **1b**.

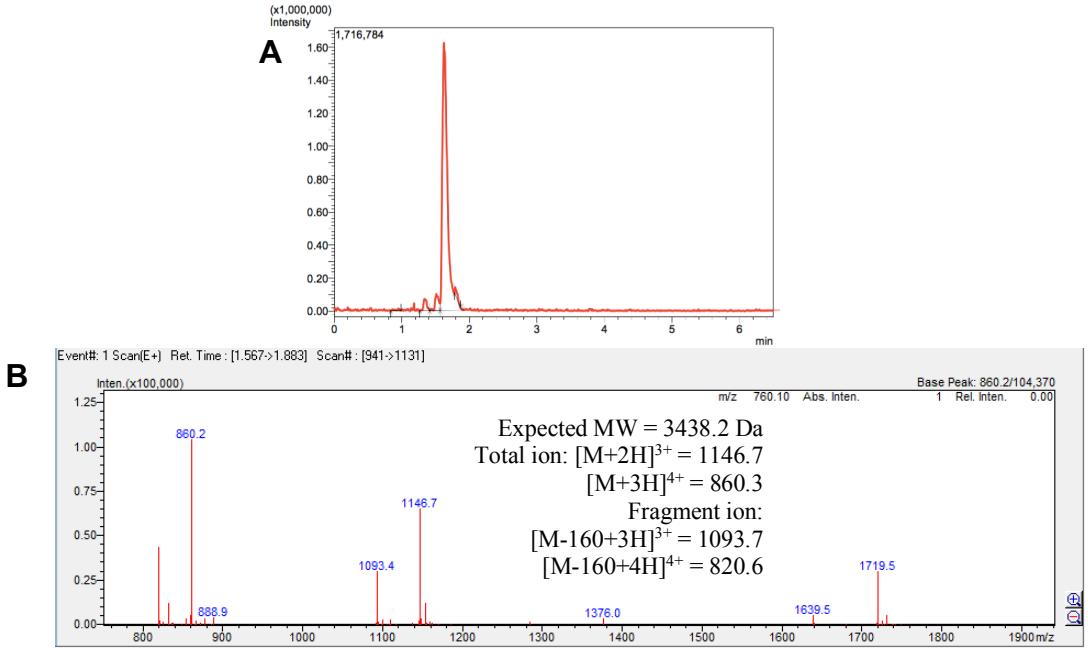
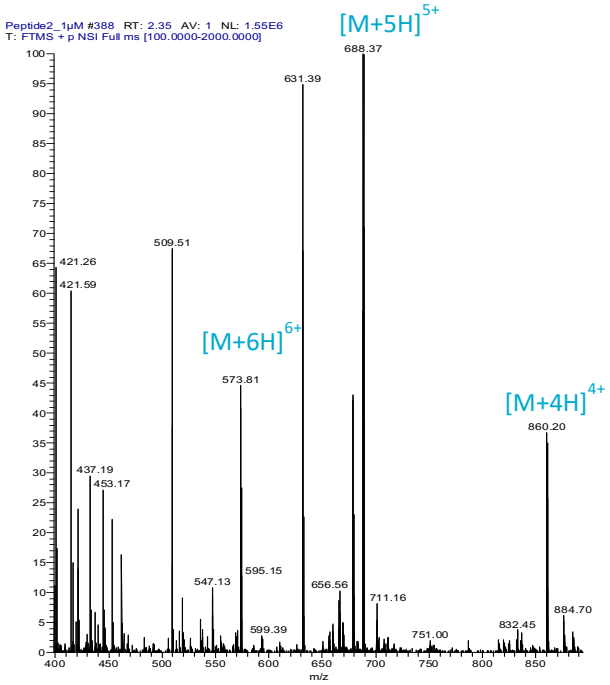


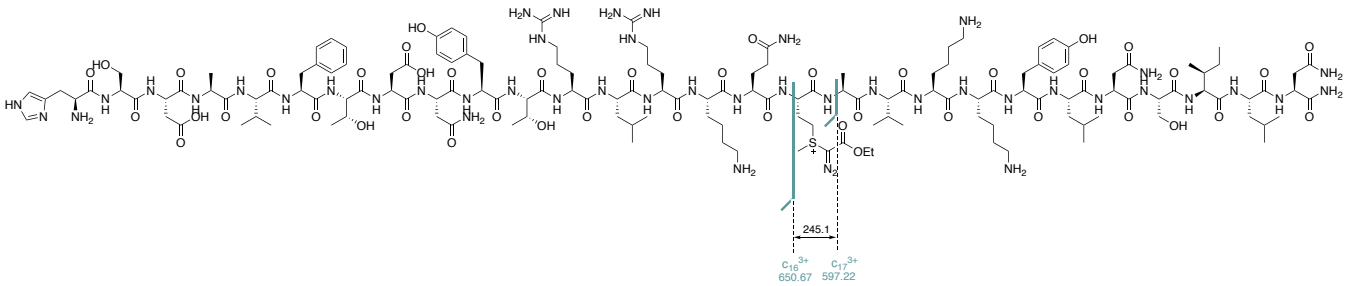
Figure S14. (**A)** Mass trace of purified **5**. **(B)** Mass spectrum of purified **5**.

MSMS Analysis of Aviptadil conjugate 5

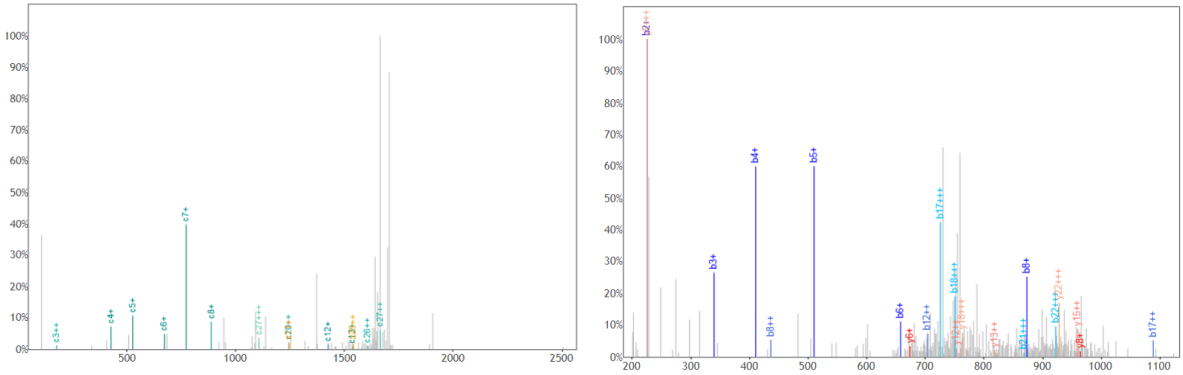


MS scan 1µM **5** in 50% ACN, 0.1% acetic acid

Figure S15: MS Scan of Aviptadil conjugate **5**



b+	b2+	b3+	c+	c2+	c3+	#	Seq	#	y+	y2+	y3+	z+	z2+	z3+
138.0662	69.5367	46.6936	155.0927	78.0500	52.3691	1	H	28						
225.0982	113.0527	75.7043	242.1248	121.5660	81.3798	2	S	27	3301.7834	1651.3953	1101.2660	3285.7647	1643.3860	1095.9264
340.1252	170.5662	114.0466	357.1517	179.0795	119.7221	3	D	26	3214.7514	1607.8793	1072.2553	3198.7326	1599.8700	1066.9157
411.1623	206.0848	137.7256	428.1888	214.5980	143.4011	4	A	25	3099.7244	1550.3658	1033.9130	3083.7057	1542.3565	1028.5734
510.2307	255.6190	170.7484	527.2572	264.1323	176.4239	5	V	24	3028.6873	1514.8473	1010.2339	3012.6686	1506.8379	1004.8944
657.2991	329.1532	219.7712	674.3257	337.6665	225.4467	6	F	23	2929.6189	1465.3131	977.2111	2913.6002	1457.3037	971.8716
758.3468	379.6770	253.4538	775.3733	388.1903	259.1293	7	T	22	2782.5505	1391.7789	928.1883	2766.5317	1383.7695	922.8488
873.3737	437.1905	291.7961	890.4003	445.7038	297.4716	8	D	21	2681.5028	1341.2550	894.5058	2665.4841	1333.2457	889.1662
987.4167	494.2120	329.8104	1004.4432	502.7252	335.4859	9	N	20	2566.4758	1283.7416	856.1635	2550.4571	1275.7322	850.8239
1150.4800	575.7436	384.1648	1167.5065	584.2569	389.8404	10	Y	19	2452.4329	1226.7201	818.1492	2436.4142	1218.7107	812.8096
1251.5277	626.2675	417.8474	1268.5542	634.7807	423.5229	11	T	18	2289.3696	1145.1884	763.7947	2273.3509	1137.1791	758.4551
1407.6288	704.3180	469.8811	1424.6553	712.8313	475.5566	12	R	17	2188.3219	1094.6646	730.1122	2172.3032	1086.6552	724.7726
1520.7128	760.8601	507.5758	1537.7394	769.3733	513.2513	13	L	16	2032.2208	1016.6140	678.0785	2016.2021	1008.6047	672.7389
1676.8139	838.9106	559.6095	1693.8405	847.4239	565.2850	14	R	15	1919.1367	960.0720	640.3838	1903.1180	952.0626	635.0442
1804.9089	902.9581	602.3078	1821.9355	911.4714	607.9833	15	K	14	1763.0356	882.0214	588.3501	1747.0169	874.0121	583.0105
1932.9675	966.9874	644.9940	1949.9940	975.5007	650.6695	16	Q	13	1634.9407	817.9740	545.6517	1618.9219	809.9646	540.3122
2177.1029	1089.0551	726.3725	2194.1294	1097.5683	732.0480	17	M	12	1506.8821	753.9447	502.9655	1490.8634	745.9353	497.6260
2248.1400	1124.5736	750.0515	2265.1665	1133.0869	755.7270	18	A	11	1262.7467	631.8770	421.5871	1246.7280	623.8676	416.2475
2347.2084	1174.1078	783.0743	2364.2349	1182.6211	788.7498	19	V	10	1191.7096	596.3584	397.9080	1175.6909	588.3491	392.5685
2475.3034	1238.1553	825.7726	2492.3299	1246.6686	831.4482	20	K	9	1092.6412	546.8242	364.8852	1076.6224	538.8149	359.5457
2603.3983	1302.2028	868.4710	2620.4249	1310.7161	874.1465	21	K	8	964.5462	482.7767	322.1869	948.5275	474.7674	316.8473
2766.4617	1383.7345	922.8254	2783.4882	1392.2477	928.5009	22	Y	7	836.4512	418.7293	279.4886	820.4325	410.7199	274.1490
2879.5457	1440.2765	960.5201	2896.5723	1448.7898	966.1956	23	L	6	673.3879	337.1976	225.1342	657.3692	329.1882	219.7946
2993.5886	1497.2980	998.5344	3010.6152	1505.8112	1004.2099	24	N	5	560.3039	280.6556	187.4395	544.2851	272.6462	182.0999
3080.6207	1540.8140	1027.5451	3097.6472	1549.3272	1033.2206	25	S	4	446.2609	223.6341	149.4252	430.2422	215.6247	144.0856
3193.7047	1597.3560	1065.2398	3210.7313	1605.8693	1070.9153	26	I	3	359.2289	180.1181	120.4145	343.2102	172.1087	115.0749
3306.7888	1653.8980	1102.9345	3323.8153	1662.4113	1108.6100	27	L	2	246.1448	123.5761	82.7198	230.1261	115.5667	77.3802
						28	N	1	133.0608	67.0340	45.0251	117.0420	59.0247	39.6855



ETD fragmentation spectrum c and z ions HCD fragmentation spectrum b and y ions

Figure S16: MSMS spectra of aviptadil conjugate **5** with the difference between c_{16}^{3+} and c_{17}^{3+} ions of 245.1 Da confirming the presence of the modification on the methionine residue.

CD Spectrum of Aviptadil conjugate 5

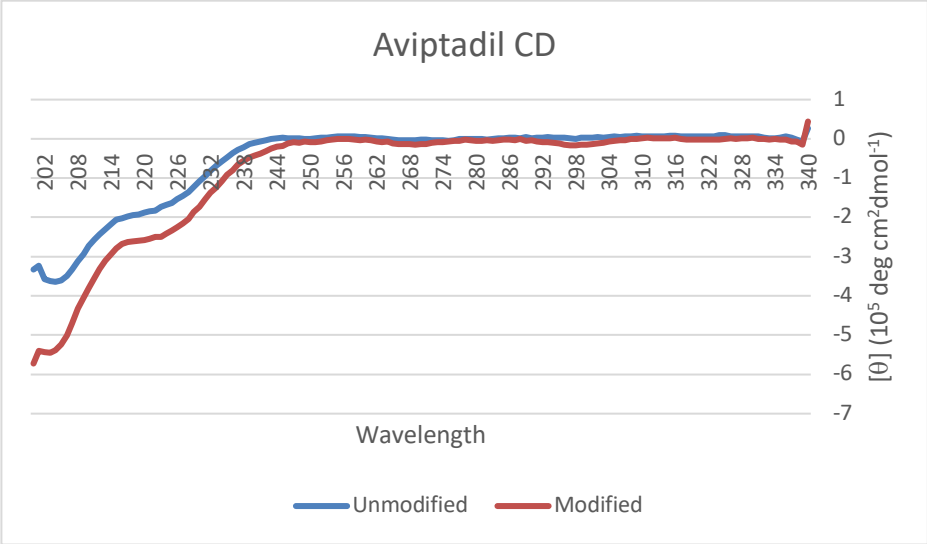
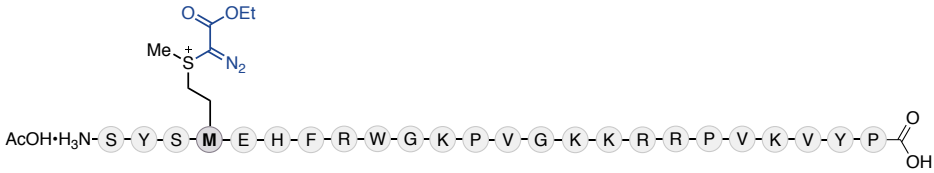


Figure S17. CD spectra of **5** (50 µM) in 5 mM pH 7.4 phosphate buffer, and Aviptadil (25 µM) in 5 mM pH 7.4 phosphate buffer.

Functionalization of Tetracosactide•OAc with 1b to form 6.



The labeling of tetracosactide was performed according to general procedure B using **1b** (100 mM in CH_3CN) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/ESI-MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S18**).

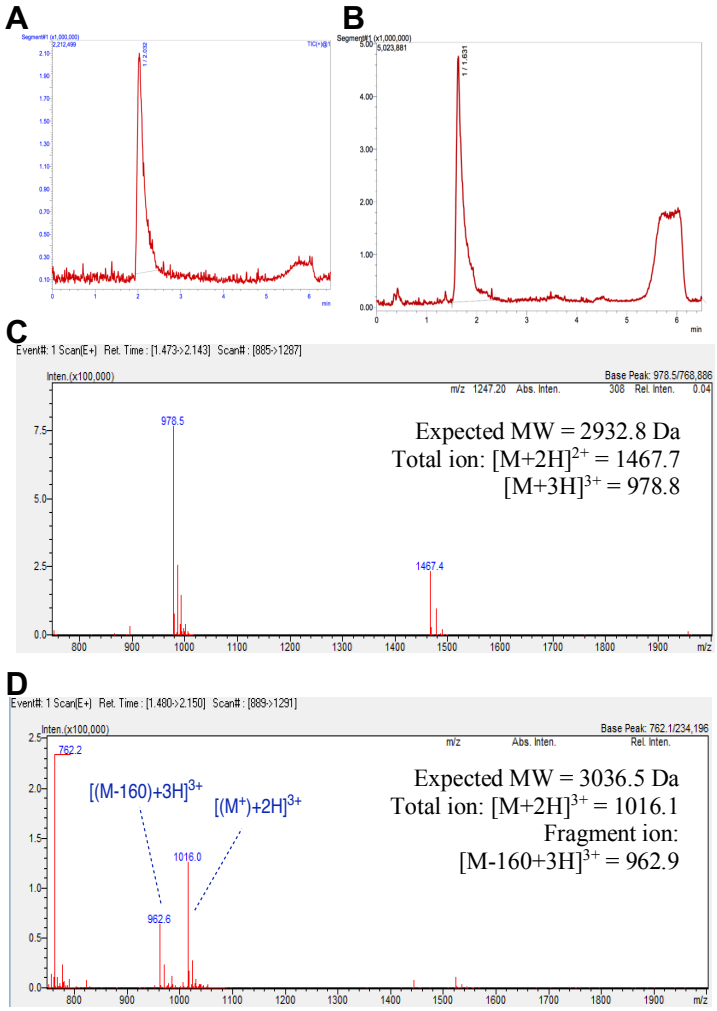


Figure S18. (**A)** Mass trace of parent Tetracosactide•OAc. **(B)** Mass trace of the crude mixture between Tetracosactide and **1b**. **(C)** Mass chromatogram of Tetracosactide•OAc (positive ionization mode, TIC from T_r=1.47-2.14 min.) **(D)** Mass chromatogram of the crude reaction to form **6** (positive ionization mode, TIC from T_r=1.48-2.15 min.)

Functionalization of Tetracosactide•OAc with 1b to form 6 in completely aqueous conditions.

The labeling of tetracosactide was performed according to general procedure B using **1b** (100 mM in H_2O) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/ESI-MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S19**).

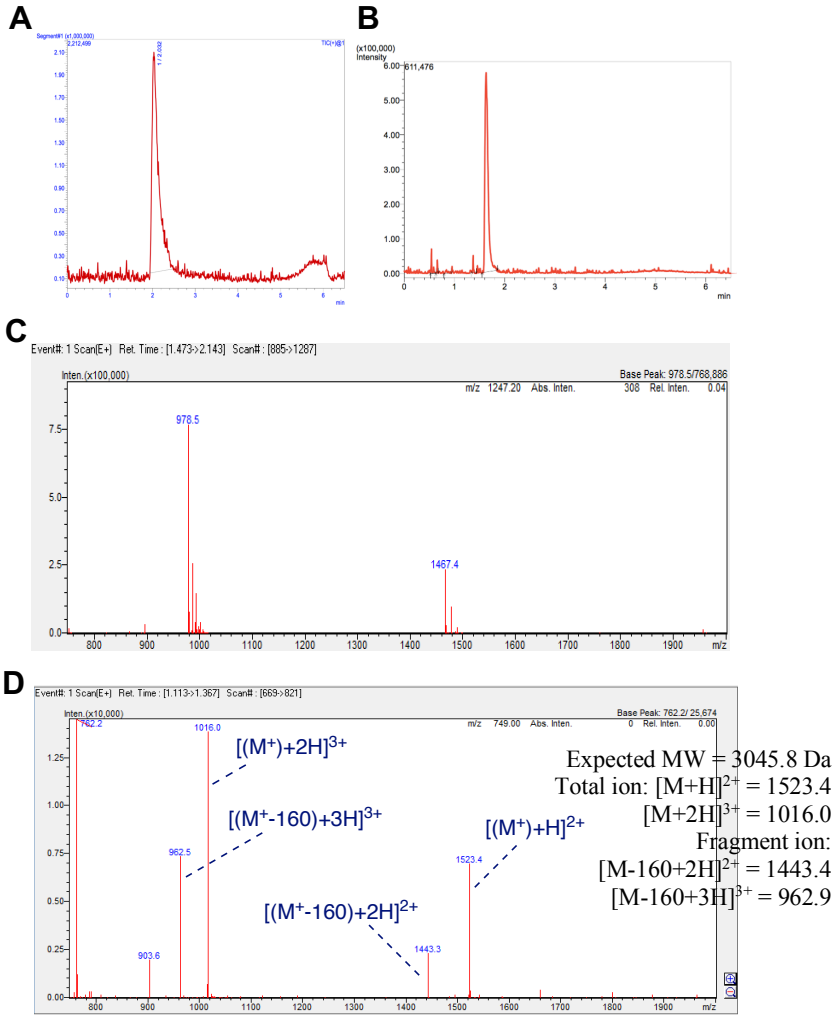


Figure S19. (**A)** Mass trace of parent Tetracosactide•OAc. **(B)** Mass trace of the crude mixture between Tetracosactide and **1b**. **(C)** Mass chromatogram of Tetracosactide•OAc (positive ionization mode, TIC from T_r=1.47-2.14 min.) **(D)** Mass chromatogram of the crude reaction to form **6** (positive ionization mode, TIC from T_r=1.11-1.37 min.)

Large Scale Synthesis of 6

A 2 mL vial equipped with a magnetic stirrer was charged with Tetracosactide•OAc (~1.8 mg, ~0.6 µmol). To the vial was added thiourea (100 mM in H_2O, 160 µL), and H_2O (120 µL). To the solution was added TEMPO (67 mM in H_2O, 120 µL) and the vial was stirred vigorously. To the stirring solution was added **1b** (100 mM in H_2O, 400 µL) and the resulting solution stirred for 5 minutes at room temperature. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. Purification *via* reverse-phase HPLC* (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95% B over 1 min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **6** as a sticky, off-white solid that was immediately redissolved in H_2O (500 µL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.0 mg/mL solution ~56% isolated yield). *Note: Purification of **6** should be carried out expediently as prolonged purification times can lead to low levels of peptide backbone hydrolysis.

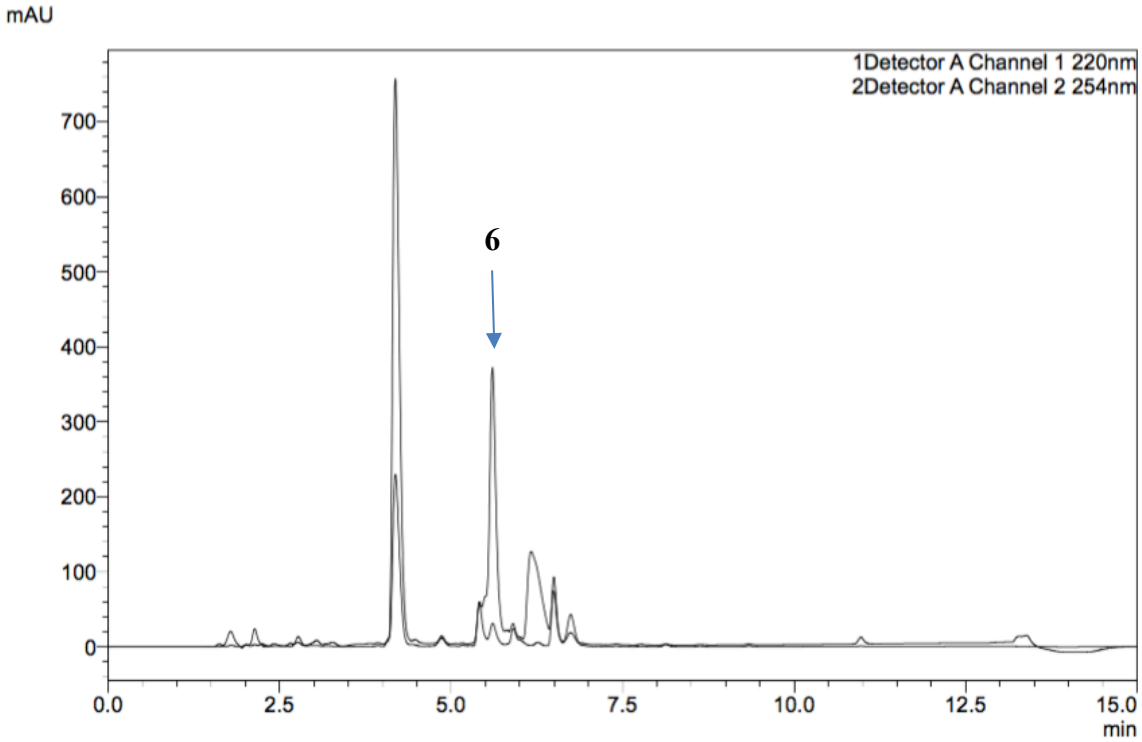


Figure S20. (**A**) HPLC trace of the crude reaction mixture of tetracosactide•OAc and **1b**

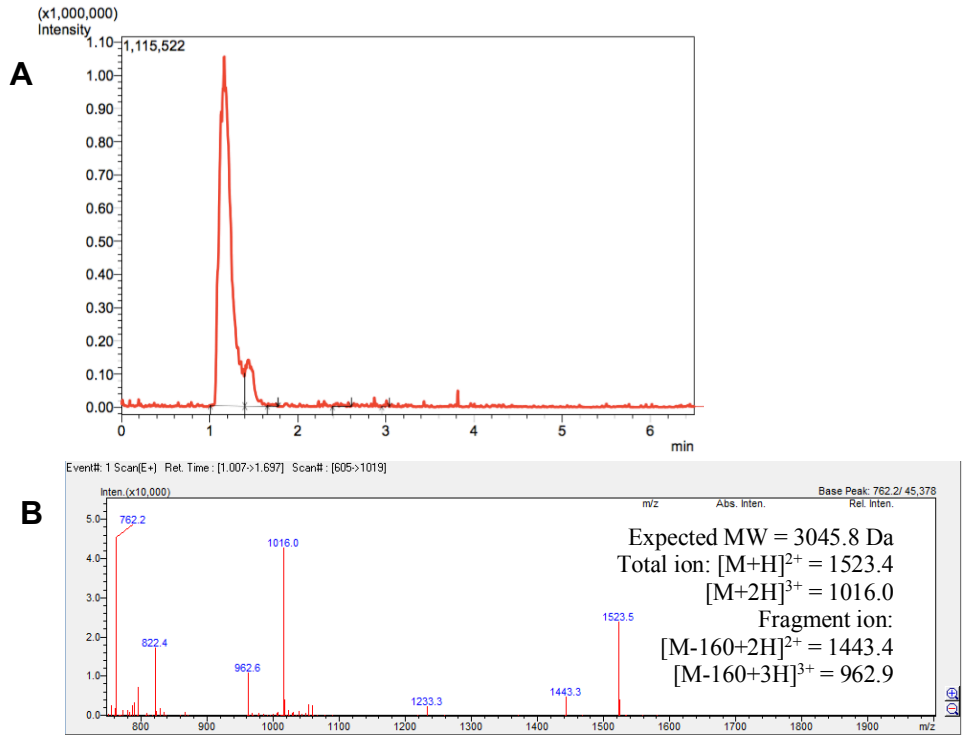
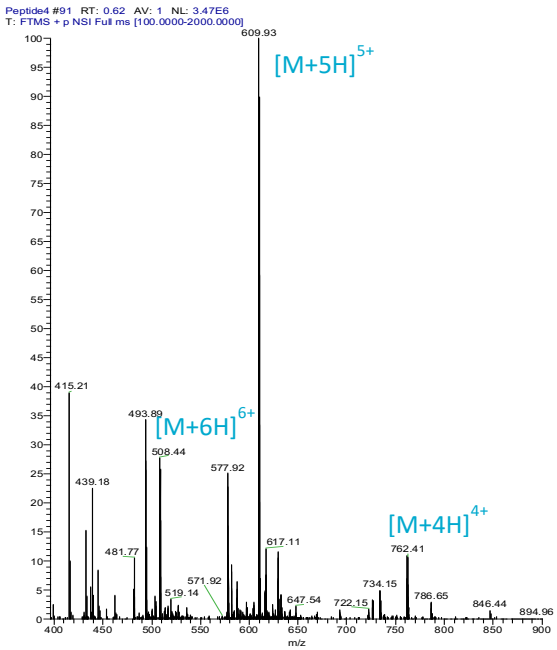


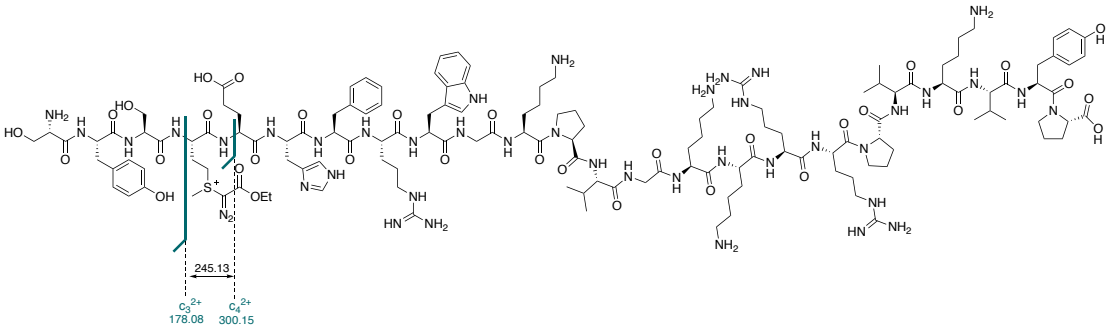
Figure S21. (**A**) Mass trace of purified **6**. **(B)** Mass spectrum of purified **6**.

MSMS Analysis of Tetracosactide 6



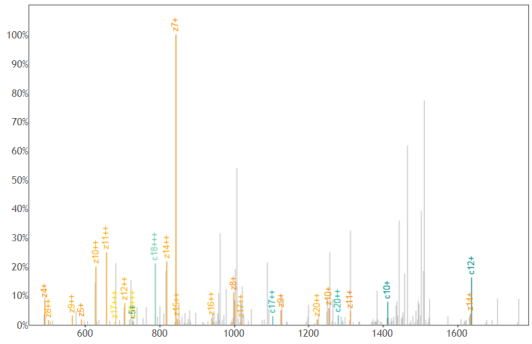
MS scan 1µM **6** in 50% ACN, 0.1% acetic

Figure S22: MS Scan of Tetracosactide conjugate **6**

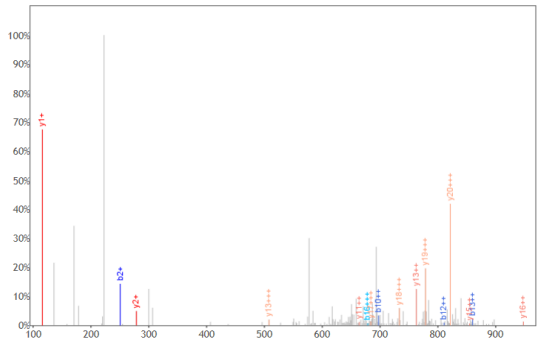


Sequence: SYS**M**EHFRWGKPVGKKRRPVKVYP

b+	b2+	b3+	c+	c2+	c3+	#	Seq	#	y+	y2+	y3+	z+	z2+	z3+
88.0393	44.5233	30.0180	105.0659	53.0366	35.6935	1	S	24						
251.1026	126.0550	84.3724	268.1292	134.5682	90.0479	2	Y	23	2958.6508	1479.8290	986.8884	2942.6321	1471.8197	981.5489
338.1347	169.5710	113.3831	355.1612	178.0842	119.0586	3	S	22	2795.5875	1398.2974	932.5340	2779.5687	1390.2880	927.1944
582.2700	291.6387	194.7615	599.2966	300.1519	200.4370	4	M	21	2708.5554	1354.7814	903.5233	2692.5367	1346.7720	898.1838
711.3126	356.1600	237.7757	728.3392	364.6732	243.4512	5	E	20	2464.4200	1232.7137	822.1449	2448.4013	1224.7043	816.8053
848.3716	424.6894	283.4620	865.3981	433.2027	289.1376	6	H	19	2335.3775	1168.1924	779.1307	2319.3587	1160.1830	773.7911
995.4400	498.2236	332.4848	1012.4665	506.7369	338.1604	7	F	18	2198.3185	1099.6629	733.4444	2182.2998	1091.6535	728.1048
1151.5411	576.2742	384.5185	1168.5676	584.7875	390.1941	8	R	17	2051.2501	1026.1287	684.4216	2035.2314	1018.1193	679.0820
1337.6204	669.3138	446.5450	1354.6469	677.8271	452.2205	9	W	16	1895.1490	948.0781	632.3879	1879.1303	940.0688	627.0483
1394.6419	697.8246	465.5521	1411.6684	706.3378	471.2277	10	G	15	1709.0697	855.0385	570.3614	1693.0510	847.0291	565.0218
1522.7368	761.8720	508.2505	1539.7634	770.3853	513.9260	11	K	14	1652.0482	826.5278	551.3543	1636.0295	818.5184	546.0147
1619.7896	810.3984	540.6014	1636.8161	818.9117	546.2769	12	P	13	1523.9533	762.4803	508.6559	1507.9346	754.4709	503.3164
1718.8580	859.9326	573.6242	1735.8845	868.4459	579.2997	13	V	12	1426.9005	713.9539	476.3050	1410.8818	705.9445	470.9654
1775.8795	888.4434	592.6313	1792.9060	896.9566	598.3069	14	G	11	1327.8321	664.4197	443.2822	1311.8134	656.4103	437.9426
1903.9744	952.4908	635.3297	1921.0010	961.0041	641.0052	15	K	10	1270.8106	635.9090	424.2751	1254.7919	627.8996	418.9355
2032.0694	1016.5383	678.0280	2049.0959	1025.0516	683.7035	16	K	9	1142.7157	571.8615	381.5767	1126.6969	563.8521	376.2372
2188.1705	1094.5889	730.0617	2205.1970	1103.1022	735.7372	17	R	8	1014.6207	507.8140	338.8784	998.6020	499.8046	333.5388
2344.2716	1172.6394	782.0954	2361.2982	1181.1527	787.7709	18	R	7	858.5196	429.7634	286.8447	842.5009	421.7541	281.5051
2441.3244	1221.1658	814.4463	2458.3509	1229.6791	820.1218	19	P	6	702.4185	351.7129	234.8110	686.3998	343.7035	229.4714
2540.3928	1270.7000	847.4691	2557.4193	1279.2133	853.1446	20	V	5	605.3657	303.1865	202.4601	589.3470	295.1771	197.1205
2668.4877	1334.7475	890.1674	2685.5143	1343.2608	895.8429	21	K	4	506.2973	253.6523	169.4373	490.2786	245.6429	164.0977
2767.5562	1384.2817	923.1902	2784.5827	1392.7950	928.8658	22	V	3	378.2023	189.6048	126.7390	362.1836	181.5954	121.3994
2930.6195	1465.8134	977.5447	2947.6460	1474.3267	983.2202	23	Y	2	279.1339	140.0706	93.7162	263.1152	132.0612	88.3766
						24	P	1	116.0706	58.5389	39.3617	100.0519	50.5296	34.0221



ETD fragmentation spectrum c and z ions



HCD fragmentation spectrum b and y ions

Figure S23: MSMS spectra of tetracosactide conjugate **6** with the difference between c_3^{2+} and c_4^{2+} ions of 245.13 Da confirming the presence of the modification on the methionine residue.

CD Spectrum of Tetracosactide 6

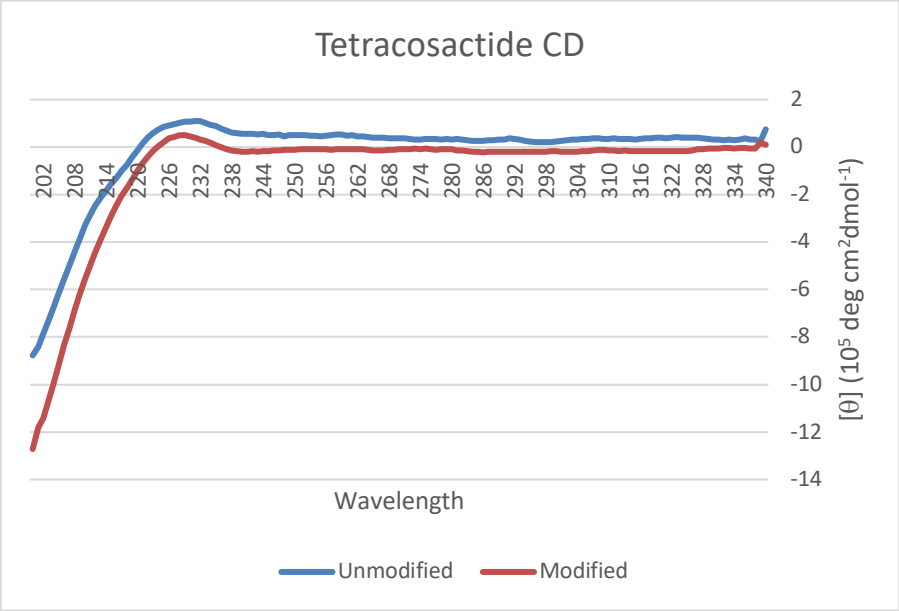
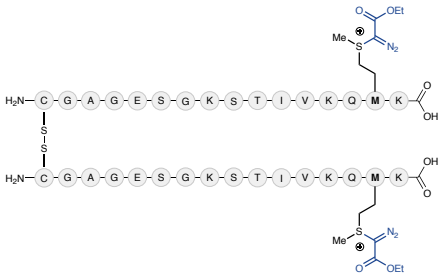


Figure S24. CD spectra of **6** (50 µM) in 5 mM pH 7.4 phosphate buffer, and Tetracosactide (25 µM) in 5 mM pH 7.4 phosphate buffer

Functionalization of GTP-binding protein fragment G-α with 1b to form 7.



A 2 mL vial equipped with a magnetic stirrer was charged with a solution containing the GTP-binding protein fragment G-α (3 mM in H_2O, 20 µL). To the vial was added thiourea (100 mM in H_2O, 40µL), formic acid (2 M, 10 µL), and H_2O (30 µL). The resulting solution was stirred vigorously. To the stirring solution was added **1b** (100 mM in H_2O, 100 µL) and the resulting solution stirred for 5 minutes. The resulting mixture was then extracted twice with diethyl ether. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion with an estimated 96% formation of **7** and 4% formation GTP-binding protein fragment+2 labels+2 oxygens (>19:1 ratio) (**Figure S25**).

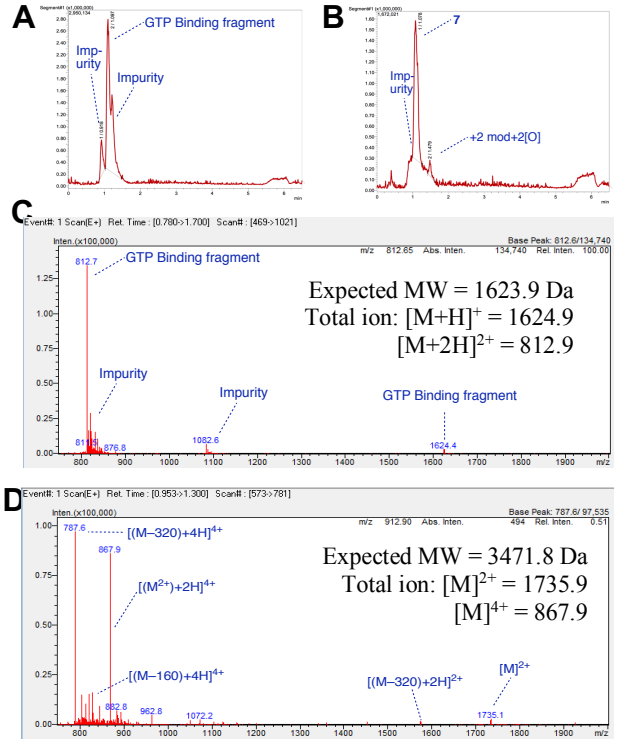
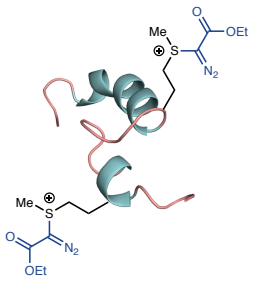


Figure S25. (**A)** Mass trace of parent GTP-binding protein fragment G-α. **(B)** Mass trace of the crude mixture between GTP-binding protein fragment G-α and **1b**. **(C)** Mass chromatogram of GTP-binding protein fragment G-α (positive ionization mode, TIC from T_r=0.8-1.7 min.) **(D)** Mass chromatogram of the crude reaction to form **7** (positive ionization mode, TIC from T_r=0.95-1.30 min.)

Functionalization of Teriparatide•OAc with 1b to form 8 in completely aqueous conditions



A 2 mL vial equipped with a magnetic stirrer was charged with a solution containing Teriparatide•OAc (1 mM in H_2O, 20 µL). To the vial was added thiourea (100 mM in H_2O, 20µL). The vial was then quickly evacuated and refilled with N_2 5 times. To the vial was added formic acid (100 mM, 10 µL) and freshly sparged water (20 µL). The vial was chilled in an ice bath. To the chilled solution was added TEMPO (33 mM in H_2O, 30 µL) and the vial was stirred vigorously. To the stirring solution was added **1b** (100 mM in H_2O, 100 µL) and the resulting solution stirred for 5 minutes at room temperature. The resulting mixture was then extracted twice with 1:1 diethyl ether/ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion (**Figure S27**).

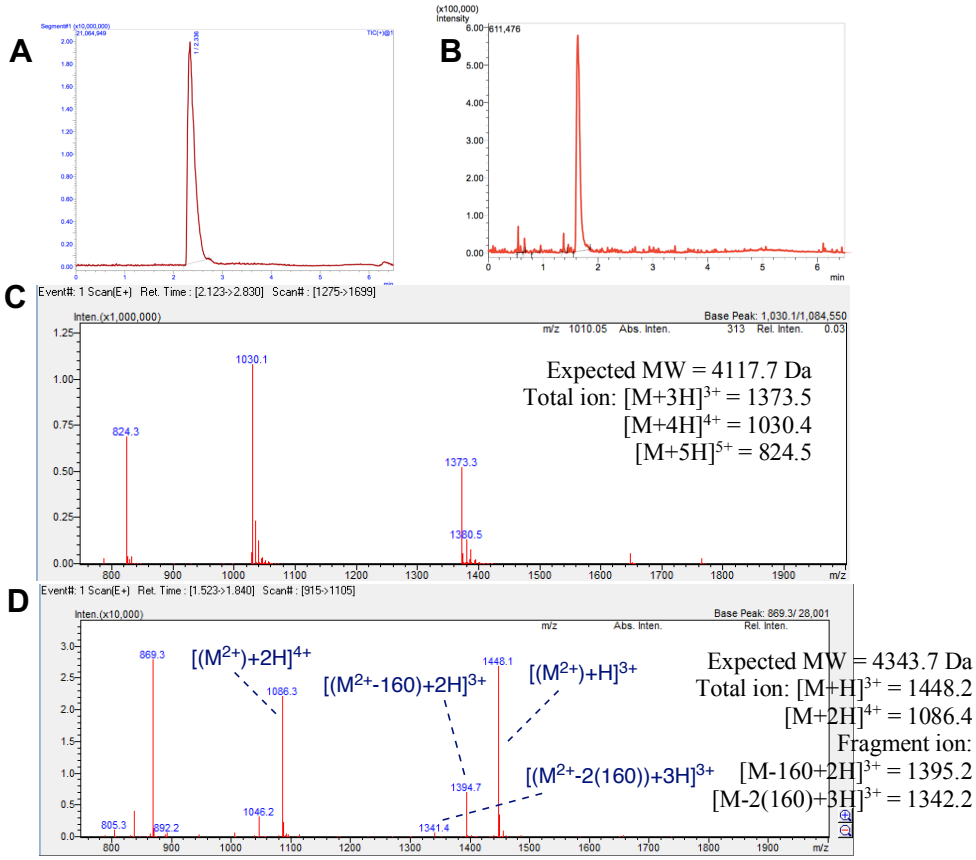


Figure S27. (**A**) Mass trace of parent Teriparatide•OAc. (**B**) Mass trace of the crude mixture between Teriparatide and **1b**. (**C**) Mass chromatogram of Teriparatide•OAc

(positive ionization mode, TIC from T_r=2.12-2.83 min.) **(D)** Mass chromatogram of the crude reaction to form **8** (positive ionization mode, TIC from T_r=1.52-1.84 min.)

Large Scale Synthesis of 8

A 2 mL vial equipped with a magnetic stirrer was charged with Teriparatide•OAc (~1.2 mg, ~0.3 µmol). To the vial was added thiourea (100 mM in H_2O, 160 µL), formic acid (0.1M in H_2O, 40 µL) and H_2O (80 µL). To the solution was added TEMPO (67 mM in H_2O, 120 µL) and the vial was stirred vigorously. To the stirring solution was added **1b** (100 mM in H_2O, 400 µL) and the resulting solution stirred for 5 minutes at room temperature. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. Purification *via* reverse-phase HPLC* (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95% B over 1 min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **8** as a sticky, off-white solid that was immediately redissolved in H_2O (500 µL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.0 mg/mL solution ~92% isolated yield). *Note: Purification of **8** should be carried out expediently as prolonged purification times can lead to low levels of peptide backbone hydrolysis.

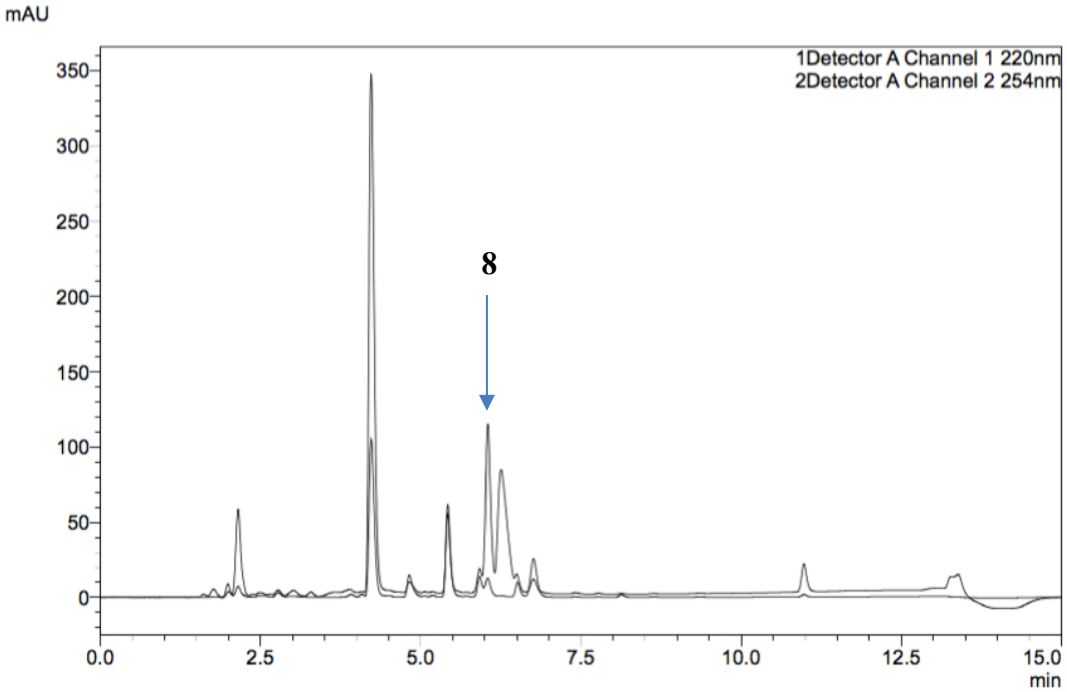


Figure S28. (**A**) HPLC trace of the crude reaction mixture of teriparatide•OAc and **1b**

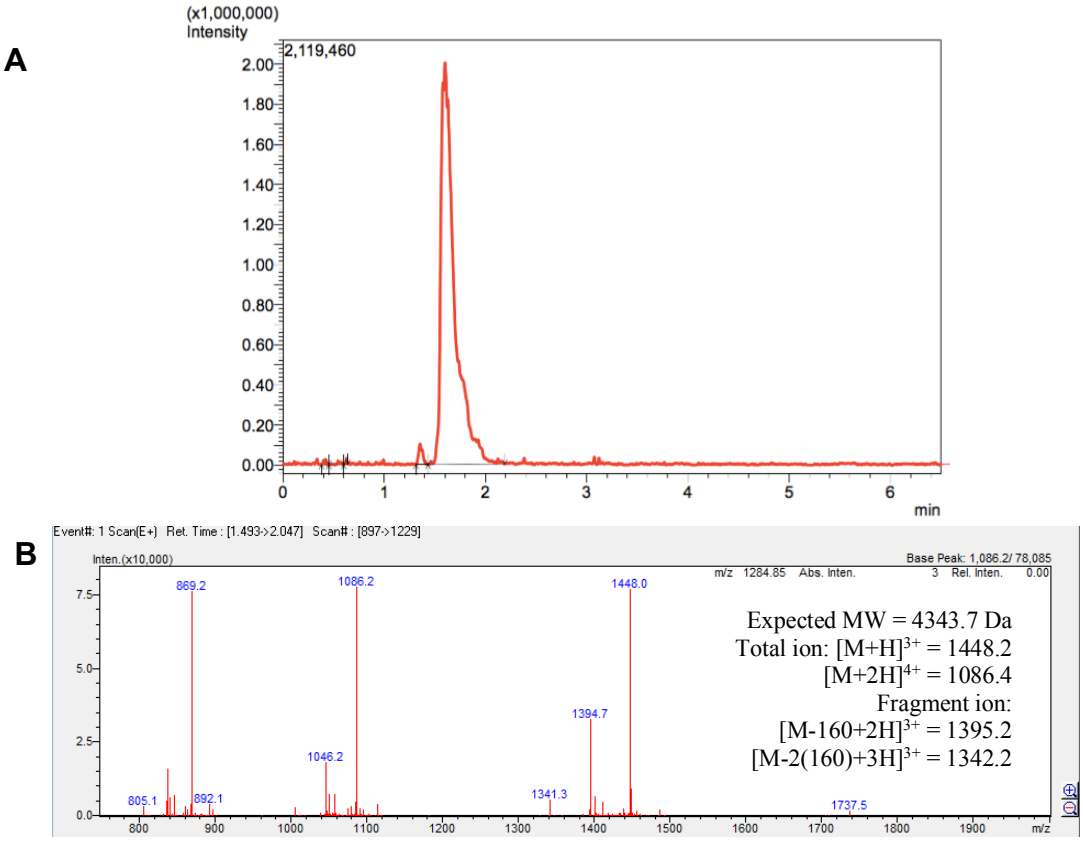
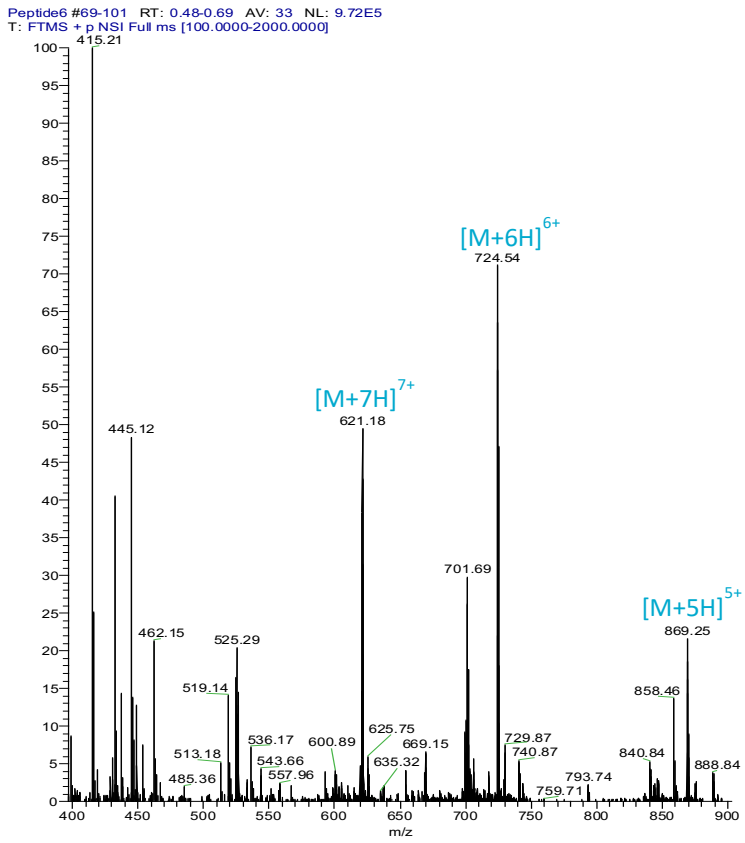


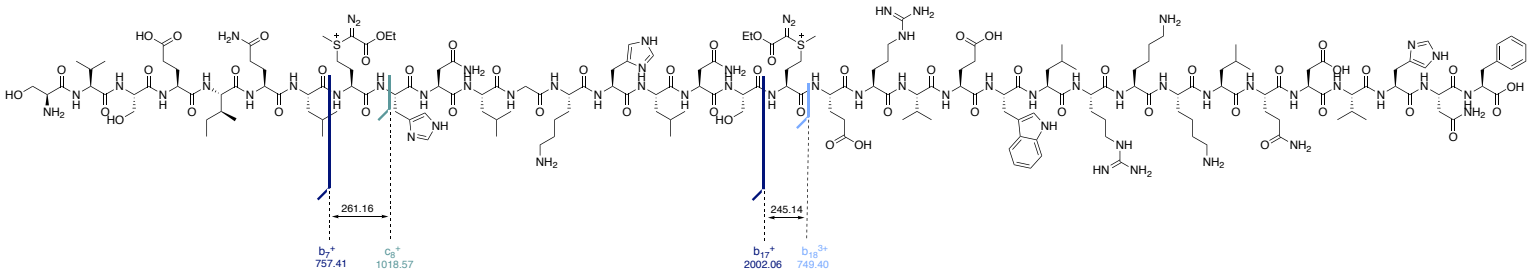
Figure S29. (**A)** Mass trace of purified **8**. **(B)** Mass spectrum of purified **8**.

MSMS Analysis of Teriparatide conjugate 8



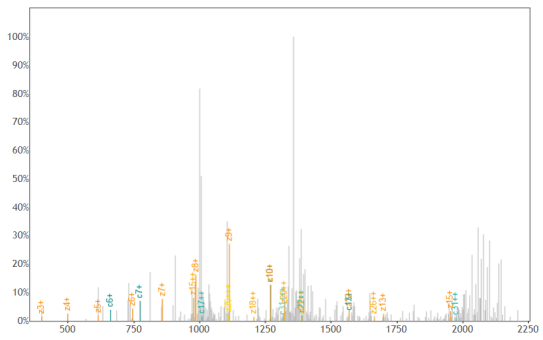
MS scan 1µM **8** in 50% ACN, 0.1% acetic acid

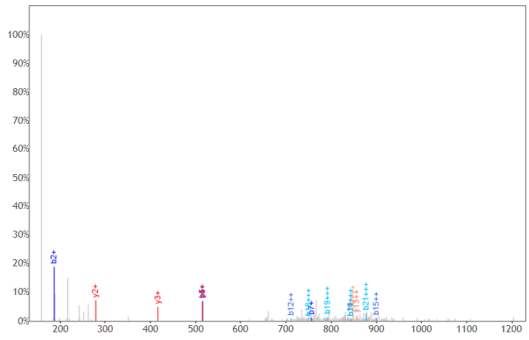
Figure S30: MS Scan of Teriparatide conjugate **8**



Sequence: SVSEIQL**M**HNLGKHLNS**M**ERVEWLRKKLEDVHNF

b+	b2+	b3+	c+	c2+	c3+	#	Seq	#	y+	y2+	y3+	z+	z2+	z3+
88.0393	44.5233	30.0180	105.0659	53.0366	35.6935	1	S	34						
187.1077	94.0575	63.0408	204.1343	102.5708	68.7163	2	V	33	4256.2800	2128.6436	1419.4315	4240.2613	2120.6343	1414.0919
274.1397	137.5735	92.0514	291.1663	146.0868	97.7269	3	S	32	4157.2116	2079.1094	1386.4087	4141.1929	2071.1001	1381.0691
403.1823	202.0948	135.0656	420.2089	210.6081	140.7411	4	E	31	4070.1796	2035.5934	1357.3980	4054.1608	2027.5841	1352.0585
516.2664	258.6368	172.7603	533.2930	267.1501	178.4358	5	I	30	3941.1370	1971.0721	1314.3838	3925.1182	1963.0628	1309.0443
644.3250	322.6661	215.4465	661.3515	331.1794	221.1220	6	Q	29	3828.0529	1914.5301	1276.6892	3812.0342	1906.5207	1271.3496
757.4090	379.2082	253.1412	774.4356	387.7214	258.8167	7	L	28	3699.9943	1850.5008	1234.0030	3683.9756	1842.4914	1228.6634
1001.5444	501.2759	334.5197	1018.5710	509.7891	340.1952	8	M	27	3586.9103	1793.9588	1196.3083	3570.8915	1785.9494	1190.9687
1138.6033	569.8053	380.2060	1155.6299	578.3186	385.8815	9	H	26	3342.7749	1671.8911	1114.9298	3326.7562	1663.8817	1109.5902
1252.6463	626.8268	418.2203	1269.6728	635.3400	423.8958	10	N	25	3205.7160	1603.3616	1069.2435	3189.6972	1595.3523	1063.9039
1365.7303	683.3688	455.9150	1382.7569	691.8821	461.5905	11	L	24	3091.6730	1546.3402	1031.2292	3075.6543	1538.3308	1025.8896
1422.7518	711.8795	474.9221	1439.7783	720.3928	480.5976	12	G	23	2978.5890	1489.7981	993.5345	2962.5703	1481.7888	988.1949
1550.8468	775.9270	517.6204	1567.8733	784.4403	523.2960	13	K	22	2921.5675	1461.2874	974.5274	2905.5488	1453.2780	969.1878
1687.9057	844.4565	563.3067	1704.9322	852.9697	568.9823	14	H	21	2793.4725	1397.2399	931.8290	2777.4538	1389.2306	926.4895
1800.9897	900.9985	601.0014	1818.0163	909.5118	606.6769	15	L	20	2656.4136	1328.7105	886.1427	2640.3949	1320.7011	880.8032
1915.0327	958.0200	639.0157	1932.0592	966.5332	644.6913	16	N	19	2543.3296	1272.1684	848.4480	2527.3109	1264.1591	843.1085
2002.0647	1001.5360	668.0264	2019.0912	1010.0493	673.7019	17	S	18	2429.2866	1215.1470	810.4337	2413.2679	1207.1376	805.0942
2246.2001	1123.6037	749.4049	2263.2266	1132.1170	755.0804	18	M	17	2342.2546	1171.6309	781.4231	2326.2359	1163.6216	776.0835
2375.2427	1188.1250	792.4191	2392.2692	1196.6382	798.0946	19	E	16	2098.1192	1049.5633	700.0446	2082.1005	1041.5539	694.7050
2531.3438	1266.1755	844.4528	2548.3703	1274.6888	850.1283	20	R	15	1969.0766	985.0420	657.0304	1953.0579	977.0326	651.6908
2630.4122	1315.7097	877.4756	2647.4387	1324.2230	883.1511	21	V	14	1812.9755	906.9914	604.9967	1796.9568	898.9820	599.6571
2759.4548	1380.2310	920.4898	2776.4813	1388.7443	926.1653	22	E	13	1713.9071	857.4572	571.9739	1697.8884	849.4478	566.6343
2945.5341	1473.2707	982.5162	2962.5607	1481.7840	988.1917	23	W	12	1584.8645	792.9359	528.9597	1568.8458	784.9265	523.6201
3058.6182	1529.8127	1020.2109	3075.6447	1538.3260	1025.8864	24	L	11	1398.7852	699.8962	466.9333	1382.7665	691.8869	461.5937
3214.7193	1607.8633	1072.2446	3231.7458	1616.3766	1077.9201	25	R	10	1285.7011	643.3542	429.2386	1269.6824	635.3448	423.8990
3342.8142	1671.9108	1114.9429	3359.8408	1680.4240	1120.6184	26	K	9	1129.6000	565.3037	377.2049	1113.5813	557.2943	371.8653
3470.9092	1735.9582	1157.6413	3487.9358	1744.4715	1163.3168	27	K	8	1001.5051	501.2562	334.5065	985.4863	493.2468	329.1670
3583.9933	1792.5003	1195.3359	3601.0198	1801.0135	1201.0115	28	L	7	873.4101	437.2087	291.8082	857.3914	429.1993	286.4686
3713.0359	1857.0216	1238.3501	3730.0624	1865.5348	1244.0257	29	E	6	760.3260	380.6667	254.1135	744.3073	372.6573	248.7740
3828.0628	1914.5350	1276.6925	3845.0894	1923.0483	1282.3680	30	D	5	631.2835	316.1454	211.0993	615.2647	308.1360	205.7598
3927.1312	1964.0692	1309.7153	3944.1578	1972.5825	1315.3908	31	V	4	516.2565	258.6319	172.7570	500.2378	250.6225	167.4174
4064.1901	2032.5987	1355.4016	4081.2167	2041.1120	1361.0771	32	H	3	417.1881	209.0977	139.7342	401.1694	201.0883	134.3946
4178.2331	2089.6202	1393.4159	4195.2596	2098.1334	1399.0914	33	N	2	280.1292	140.5682	94.0479	264.1105	132.5589	88.7083
						34	F	1	166.0863	83.5468	56.0336	150.0675	75.5374	50.6940





ETD fragmentation spectrum c and z ions

HCD fragmentation spectrum b and y ions

Figure S31: MSMS spectra of teriparatide conjugate **8** with the difference between b_7^+ and c_8^+ ions of 261.2 Da and difference between b_{17}^+ and b_{18}^{3+} ions of 245.1 Da confirming the presence of the modification on the methionine residues.

CD Spectrum of Teriparatide conjugate 8

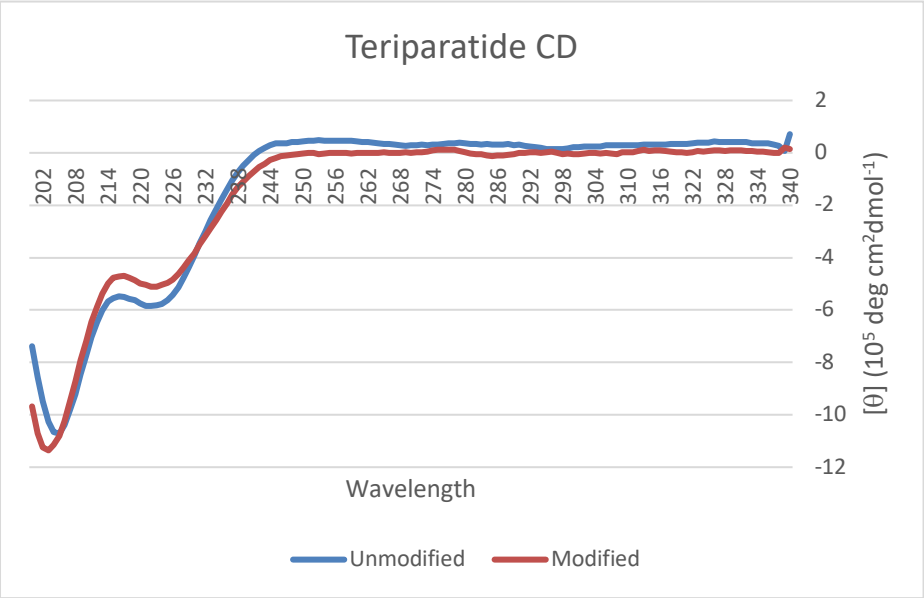
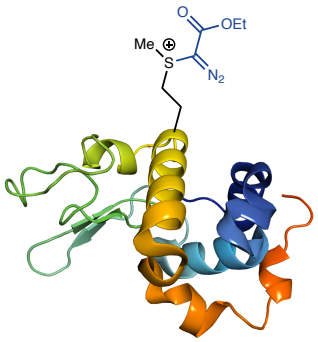


Figure S32. CD spectra of **8** (50 µM) in 5 mM pH 7.4 phosphate buffer, and Tetracosactide (25 µM) in 5 mM pH 7.4 phosphate buffer

*In contrast to the polypeptide sulfonium conjugates, MS/MS analysis was not possible with the protein sulfonium conjugates **9**, **10** & **11**, despite our best efforts. The presence of the sulfonium ion seems to interfere with both the digest and the MS/MS analysis in these larger systems, rendering the data uninterpretable. Efforts to understand the effect of the sulfonium ion are ongoing.*

Functionalization of Lactalbumin with 1b to form 9.



A 2 mL vial equipped with a magnetic stirrer was evacuated and refilled with N_2. To the vial was added an N_2-sparged solution containing Lactalbumin (350 µM in 0.1M thiourea (aq), 20 µL), an N_2-sparged solution of thiourea (0.1M in H_2O, 20µL), a solution of dilute HCl (100 mM, 20 µL) and N_2-sparged H_2O (30 µL). The vial was chilled in an ice bath. To the chilled solution was added an N_2-sparged solution of TEMPO (100 mM in 1:1 H_2O: CH_3CN, 20 µL) and the vial was stirred vigorously. To the stirring solution was added the iodonium salt (100 mM in CH_3CN, 100 µL) and the resulting solution stirred for 1 minute at 0° C. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS, (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min), and was judged to have proceeded in 88% conversion with a >10:1 ratio of single:double modification (**Figure S33**).

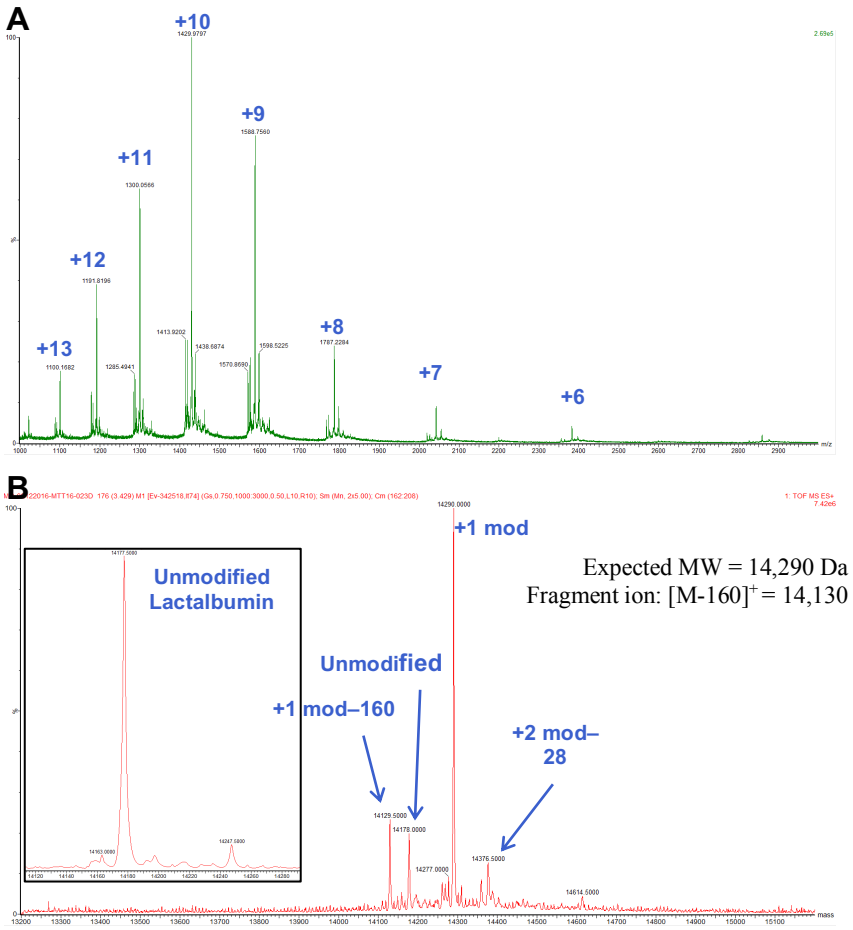
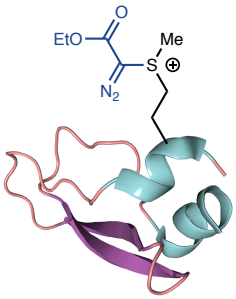


Figure S33. **(A)** Mass chromatogram of the crude reaction mixture between Lactalbumin and **1b** to form **9**. **(B)** Deconvoluted mass spectrum of the crude reaction to form **9**. The insert depicts the deconvoluted mass spectrum of unmodified Lactalbumin.

Functionalization of Aprotinin with 1b to form 10.



The labeling of Aprotinin was performed according to general procedure B using **1b** (100 mM in H_2O) and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS (C4 column (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min), and was judged to have proceeded in >95% conversion, with a 3:1 ratio of **10**:aprotinin sulfoxide observed (**Figure S34**).

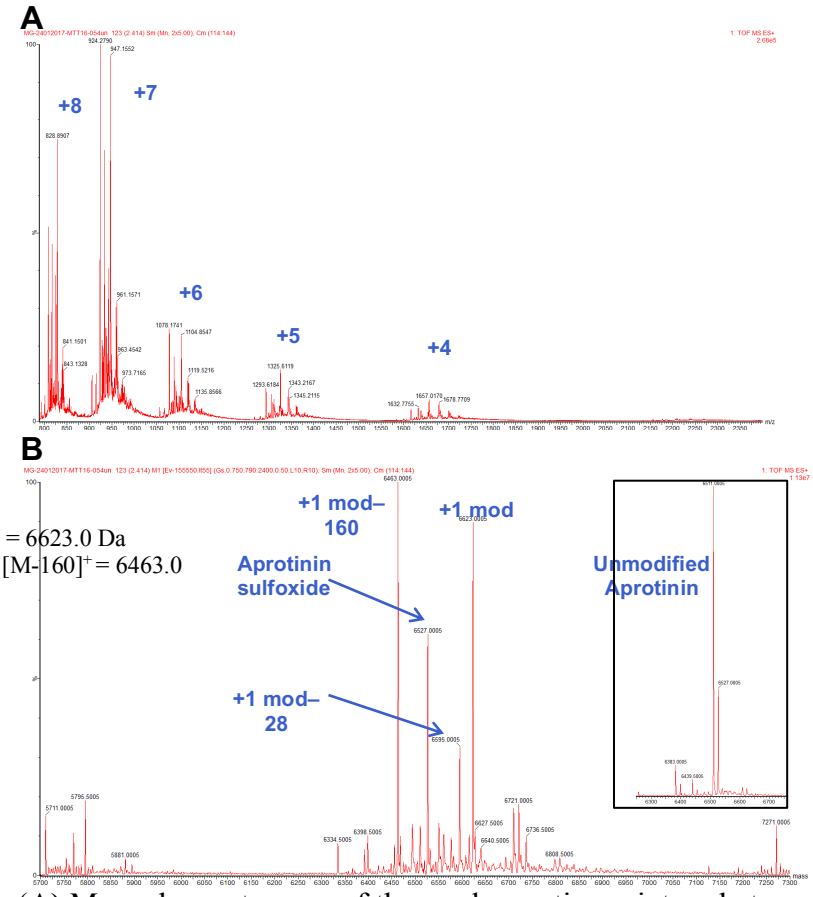
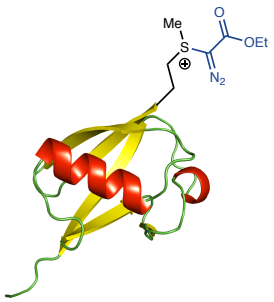


Figure S34. **(A)** Mass chromatogram of the crude reaction mixture between Aprotinin and **1b** to form **10**. **(B)** Deconvoluted mass spectrum of the crude reaction to form **10**. The insert depicts the deconvoluted mass spectrum of unmodified Aprotinin.

Functionalization of Ubiquitin with 1b to form 11.



A 2 mL vial equipped with a magnetic stirrer was evacuated and refilled with N_2. To the vial was added an N_2-sparged solution containing Ubiquitin (1 mM in 0.1M thiourea (aq), 20 µL), an N_2-sparged solution of thiourea (100 mM in H_2O, 20µL), formic acid (100 mM, 10 µL) and N_2-sparged H_2O (50 µL), and the resulting solution was stirred vigorously. To the stirring solution was added **1b** (100 mM in CH_3CN, 100 µL) and the resulting solution stirred for 5 minutes. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS, (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min) and was judged to have proceeded in 88% conversion with a **11**:double modification ratio of 10:1 (**Figure S35**).

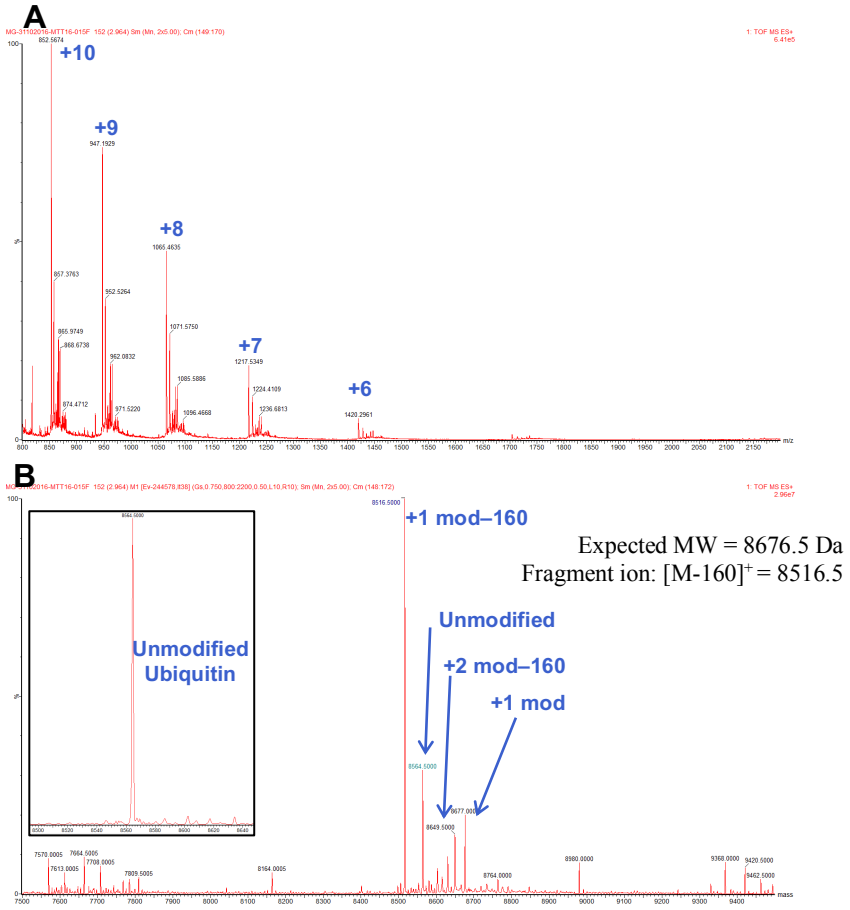


Figure S35. (**A)** Mass chromatogram of the crude reaction mixture between Ubiquitin and **1b** to form **11**. **(B)** Deconvoluted mass spectrum of the crude reaction to form **11**. The insert depicts the deconvoluted mass spectrum of unmodified Ubiquitin.

Large Scale Synthesis of 11

This larger scale reaction was run to partial completion in order to exemplify the purification of starting Ubiquitin and diazoester conjugate **11*

A 2 mL vial equipped with a magnetic stirrer was charged with Ubiquitin (~1.5 mg, ~0.2 µmol). To the vial was added thiourea (100 mM in H_2O, 100 µL), formic acid (0.1M in H_2O, 25 µL) and H_2O (125 µL). To the stirring solution was added **1b** (100 mM in H_2O, 250 µL) and the resulting solution stirred for 5 minutes at room temperature. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. **11** was purified by ion exchange chromatography (MonoS 4.6/100 PE column, GE Healthcare) using a NaCl gradient (0-1 M) in 50 mM ammonium acetate pH 4.5 on an ÄKTAexplorer (Pharmacia Biotech), and fractions directly analysed my MS.

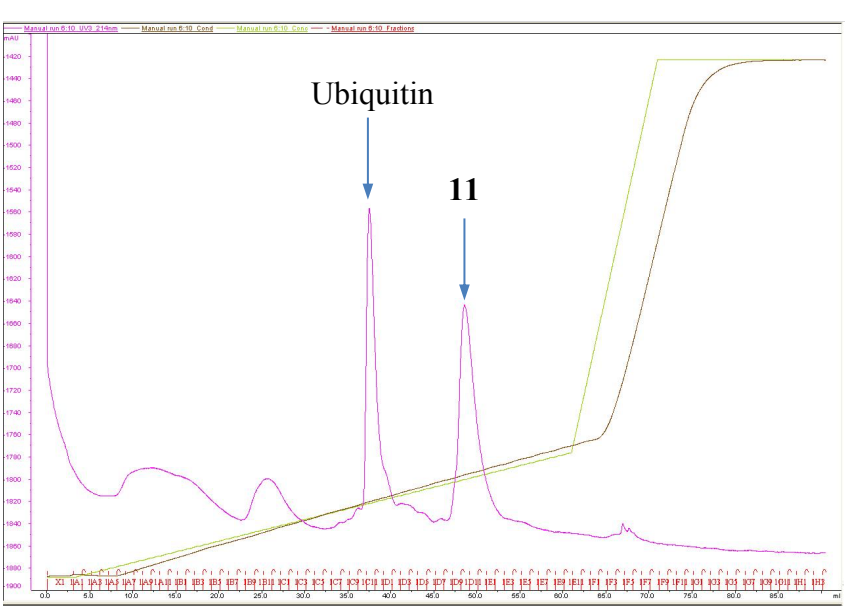


Figure S36: FPLC trace of Ubiquitin conjugate **11**, purified using MonoS 4.6/100 PE column, GE Healthcare. Clear separation of unmodified Ubiquitin and modified conjugate **11** was achieved due to the differing elution times of the charged species **11** and uncharged, unmodified ubiquitin using an ion exchange column. Diagnostic fragment ion of [Ub+mod-160] MW = 8516.5 was observed in the product peak of **11**. Hence, this indicates that the diagnostic fragmentation must be an artefact of the mass spectrometer, as proposed, otherwise the uncharged resulting species would have a dramatically different retention time using this ion exchange purification method.

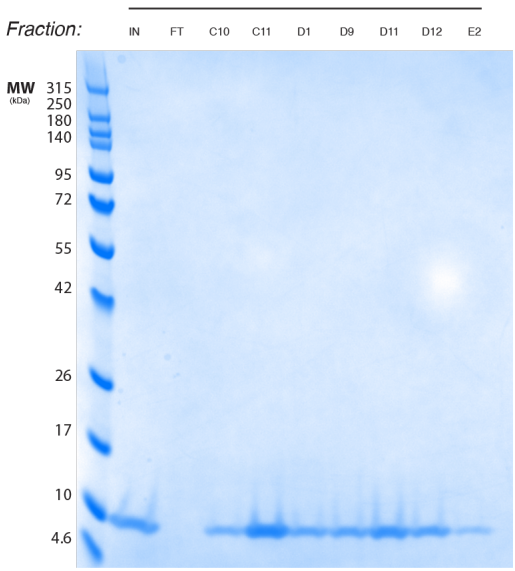
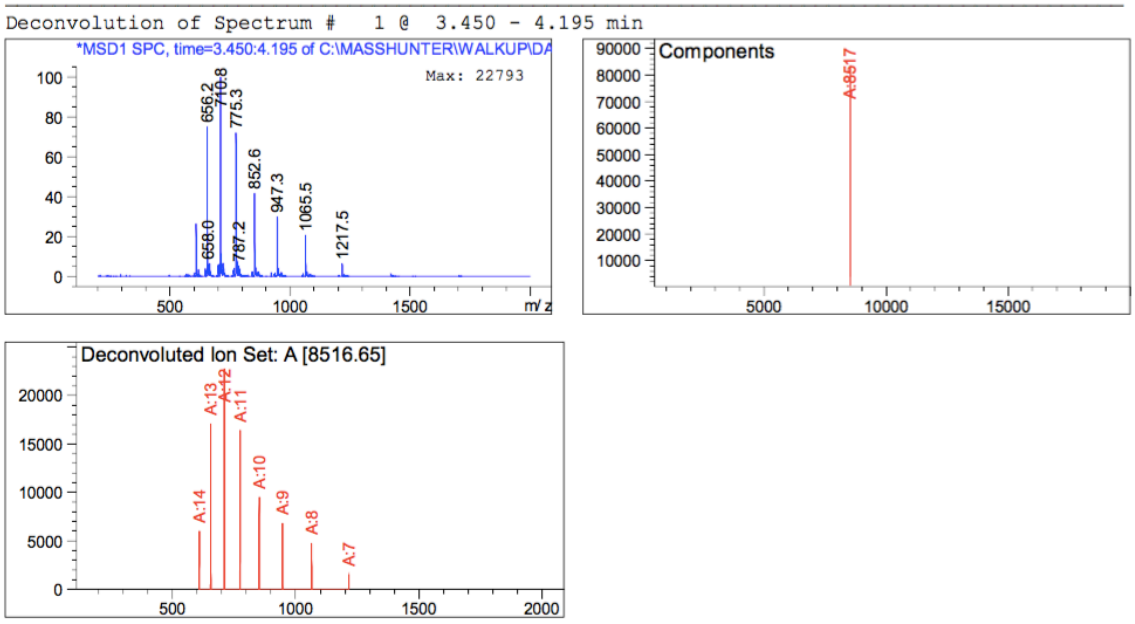
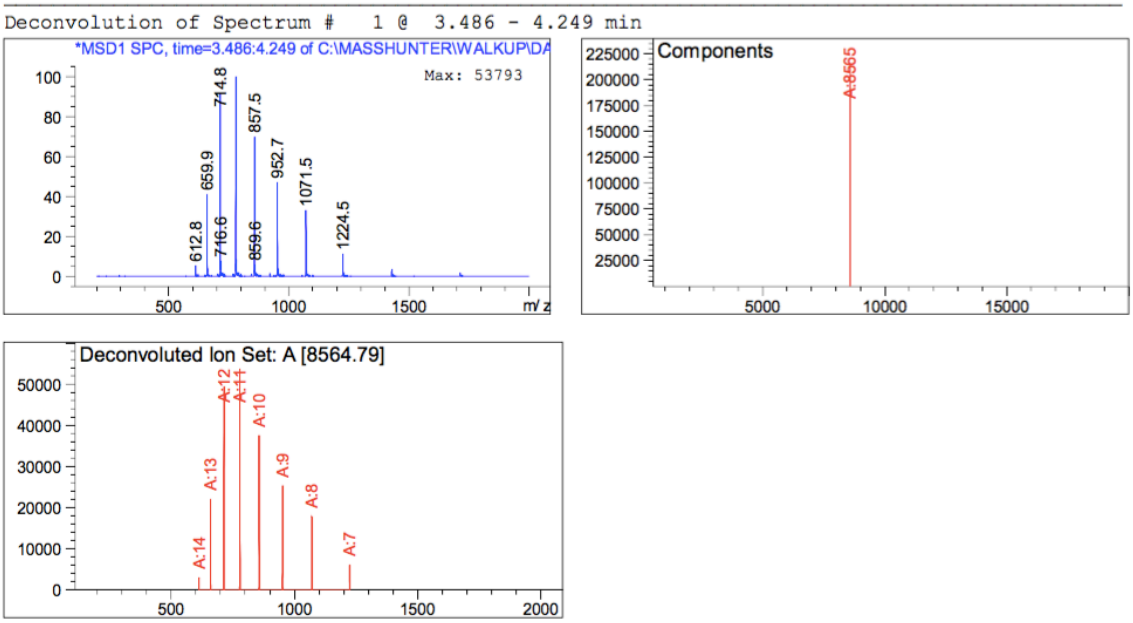


Figure S37: Gel of purified Ubiquitin fractions from FPLC purification run with Bolt 4-12% Bis-Tris gels (Thermo Scientific) and were run for 30 min at 165V (constant) using MES buffer (Thermo Scientific). The ladder on the gel is ProSieve QuadColor protein marker (4.6 kDa – 300 kDa; Lonza) and the gel was stained using InstantBlue (Generon)



Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8516.65	83581	100.00

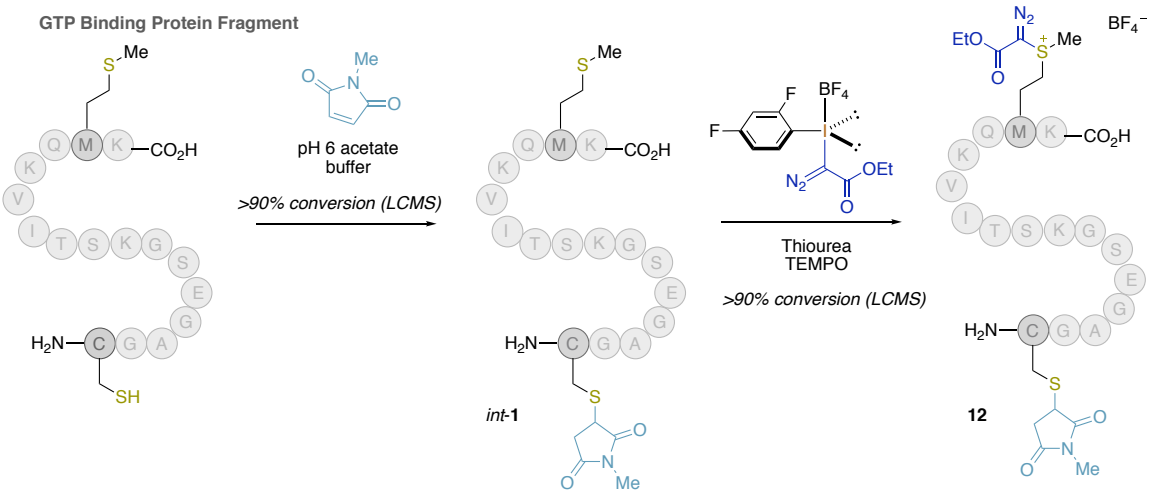
Figure S38: Protein LCMS and deconvoluted MS of purified fraction of **11** from IEX column, showing fragment ion of **11**-160. Were this the iminolactone byproduct, this would likely have hydrolysed to the homoserine lactone and would have eluted at a dramatically different retention time on IEX, similar to that of the parent ubiquitin.



Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8564.79	214144	100.00

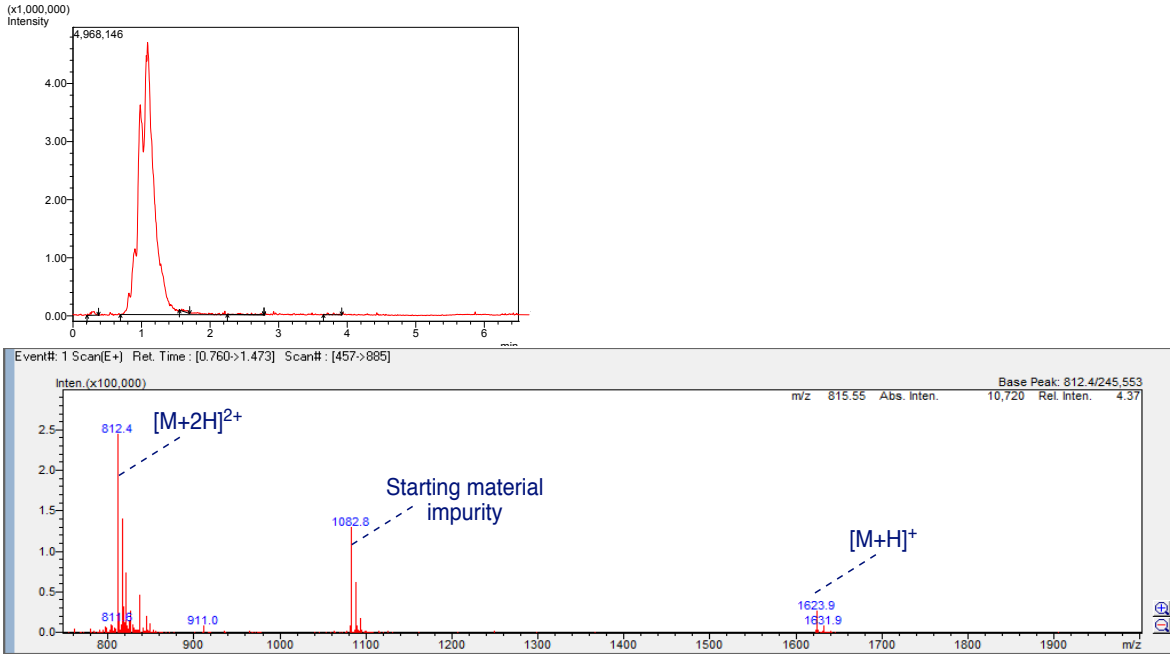
Figure S39: Protein LCMS and deconvoluted MS of purified ubiquitin.

Functionalization of GTP Binding Protein Fragment-Gα with 1b to form 12.

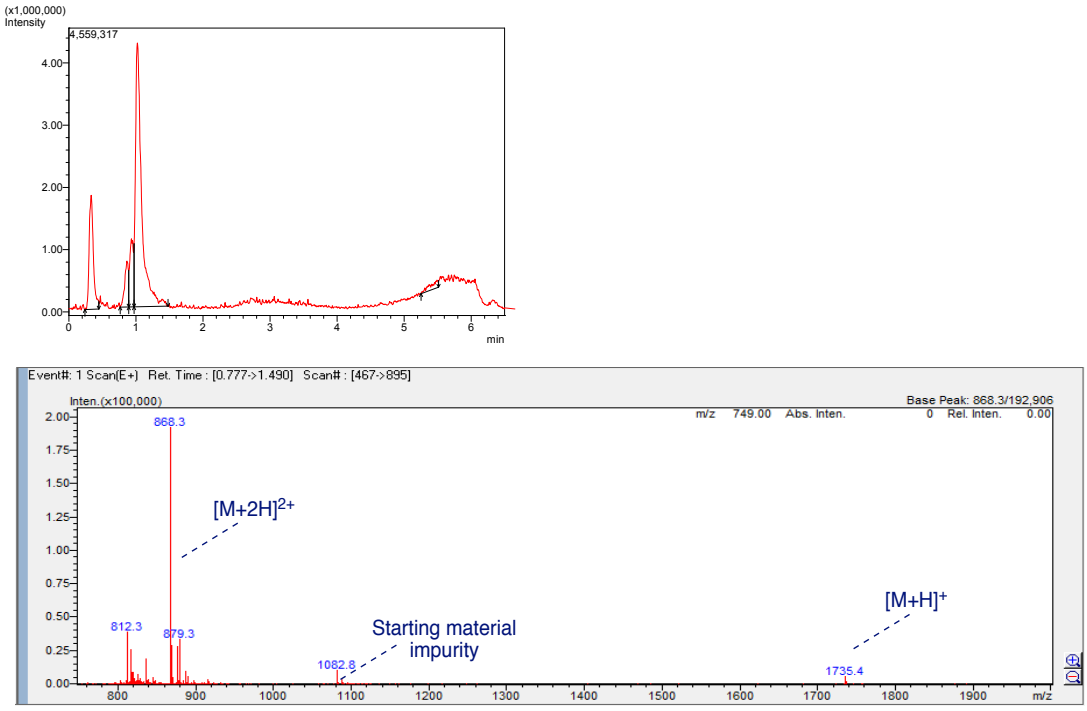


A 2 mL vial equipped with a magnetic stirrer was charged with a solution of GTP Binding Protein Fragment α (1 mM in pH 6 buffer, 50 μL). To the vial was added a solution of N-methyl maleimide (10 mM in MeCN, 50 μL) and the resulting solution stirred vigorously for 30 minutes at room temperature. The resulting solution was then analyzed directly *via* LC/MS C18 column ((150 x 10 mm, 10 μm), gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 mL/min), and was judged to have proceeded in >90% conversion in relation to starting material (Figure **S20**). A 2 mL vial equipped with a magnetic stirrer was charged with the crude reaction solution (500 μM in 1:1 pH6 buffer:MeCN, 100 μL). To the vial was added thiourea (100 mM in H_2O, 30 μL). To the resulting solution was added TEMPO (67 mM in H_2O, 30 μL) immediately followed by iodonium salt **1b** (250 mM in H_2O, 40 μL) and the reaction stirred vigorously for 2 minutes at room temperature. The resulting mixture was extracted twice with ethyl acetate:diethyl ether (1:1). The remaining organic volatiles were removed from the aqueous layer using a rotary evaporator, and the aqueous layer analysed directly *via* LC/MS C18 column ((150 x 10 mm, 10 μm), gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 mL/min), and was judged to have proceeded in >90% conversion in relation to starting material (Figure **S40**).

A


(x1,000,000)
Intensity
4,968,146
4.00
3.00
2.00
1.00
0.00
0
1
2
3
4
5
6
min
Event#: 1 Scan(E+) Ret. Time : [0.760->1.473] Scan# : [457->885]
Inten.(x100,000)
Base Peak: 812.4/245,553
m/z 815.55 Abs. Inten. 10,720 Rel. Inten. 4.37
812.4
[M+2H]2+
Starting material
impurity
1082.8
[M+H]+
1623.9
1631.9
911.0
2.5
2.0
1.5
1.0
0.5
0.0
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z

B


(x1,000,000)
Intensity
4,559,317
4.00
3.00
2.00
1.00
0.00
0
1
2
3
4
5
6
min
Event#: 1 Scan(E+) Ret. Time : [0.777->1.490] Scan# : [467->895]
Inten.(x100,000)
Base Peak: 868.3/192,906
m/z 749.00 Abs. Inten. 0 Rel. Inten. 0.00
868.3
[M+2H]2+
812.3
879.3
Starting material
impurity
1082.8
[M+H]+
1735.4
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z

C

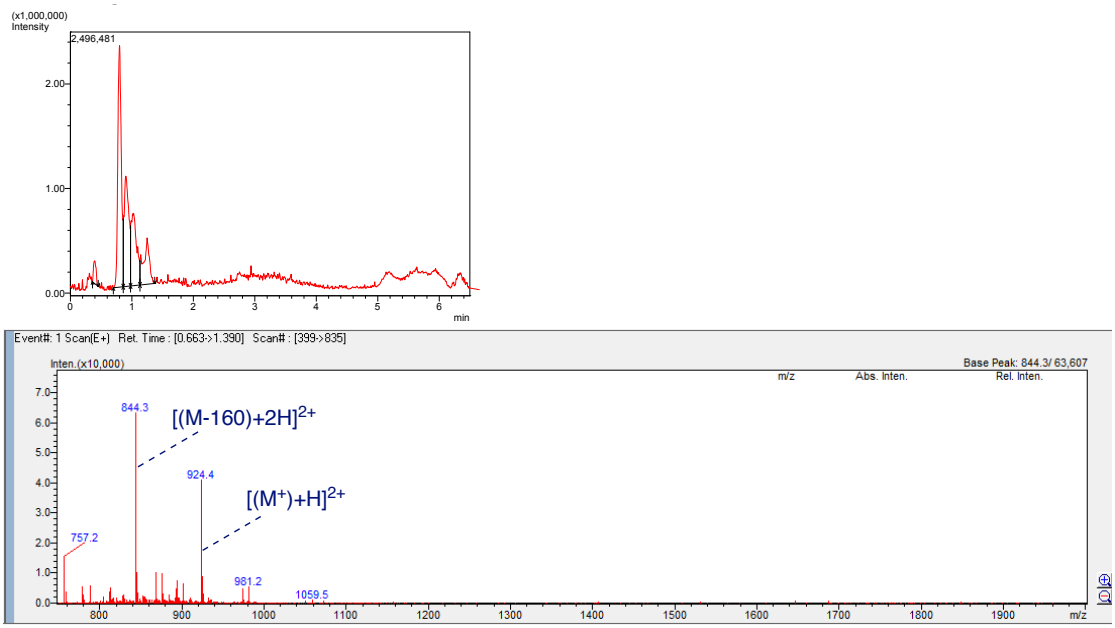
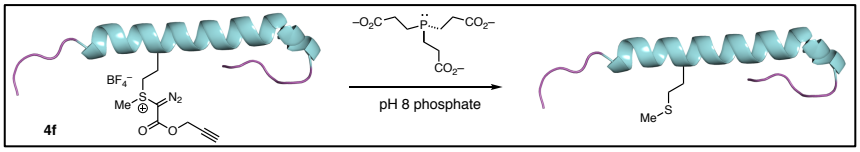


Figure S40: (A) Chromatogram and mass trace of parent GTP Binding Protein Fragment α - estimated at ~75% purity. **(B)** Chromatogram and mass trace of intermediate ***int*-1** from crude reaction of GTP Binding Protein Fragment-Gα and N-methyl maleimide**. (C)** Chromatogram and mass trace of crude reaction to form **12**

Phosphine promoted “de-ligation” of α-sulfoniumdiazoesters

Phosphine promoted “De–ligation” of conjugate 4f.



A vial was charged with a solution containing sulfonium conjugate **4f** (600 µM, 20 µL). To the vial was added a solution of Tris-(2-carboxyethyl) phosphine (1.3 mM in pH 8 phosphate buffer, 80 µL), and vial was allowed to stand for 30 minutes. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation reversal was judged to have proceeded in 90% conversion. A similar reaction ([**4f**]= 64 µM) was performed in pH 3 formic acid buffer, which slowed the rate of conjugate hydrolysis, allowing for the observation of the formation of reduced **4f’** (**Figure S41**).

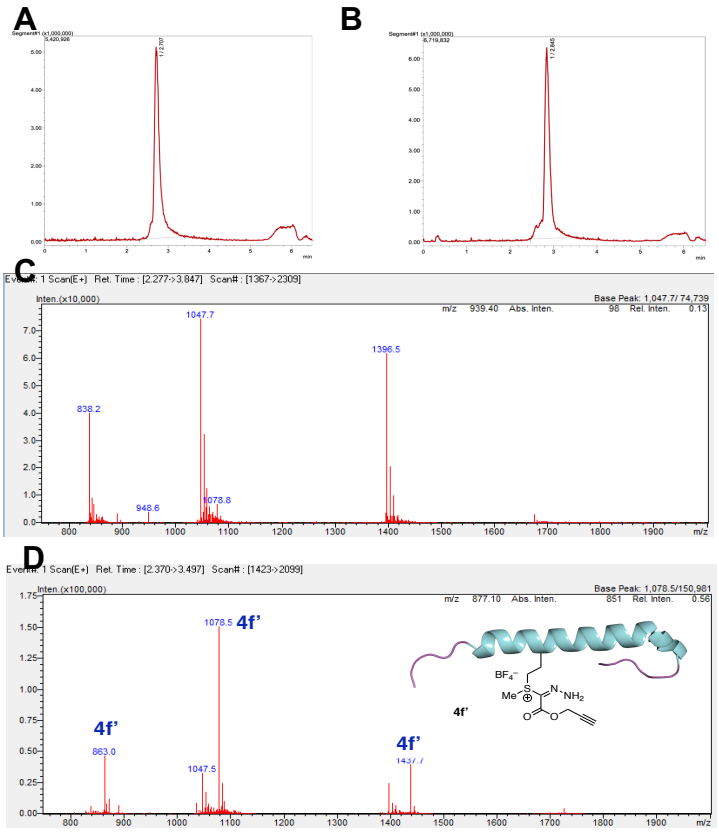
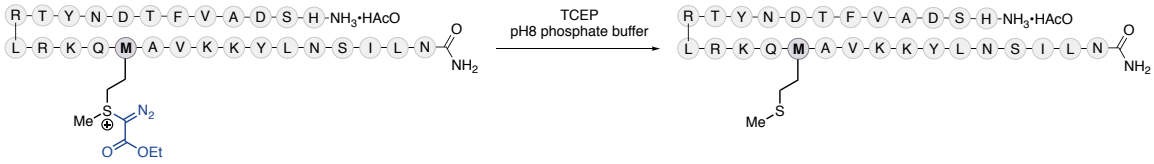


Figure S41. (**A**) Mass trace of **4f**. **(B)** Mass trace of the crude mixture between **4f** and TCEP at pH 8. **(C)** Mass spectrum of the crude mixture between **4f** and TCEP at pH 8 (positive ionization mode, TIC from T_r=2.78-3.85 min.) **(D)** Mass spectrum of the crude mixture between **4f** and TCEP at pH 3 (positive ionization mode, TIC from T_r=2.37-3.50 min).

Phosphine promoted "De–ligation" of aviptadil conjugate 5



A vial was charged with a solution containing sulfonium conjugate **5** (750 µM, 25 µL). To the vial was added a solution of Tris-(2-carboxyethyl) phosphine (1.7 mM in pH 8 phosphate buffer, 50 µL) and H_2O (25 µL) and vial was allowed to stand for 30 minutes. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation reversal was judged to have proceeded in >95% conversion (**Figure S42**).

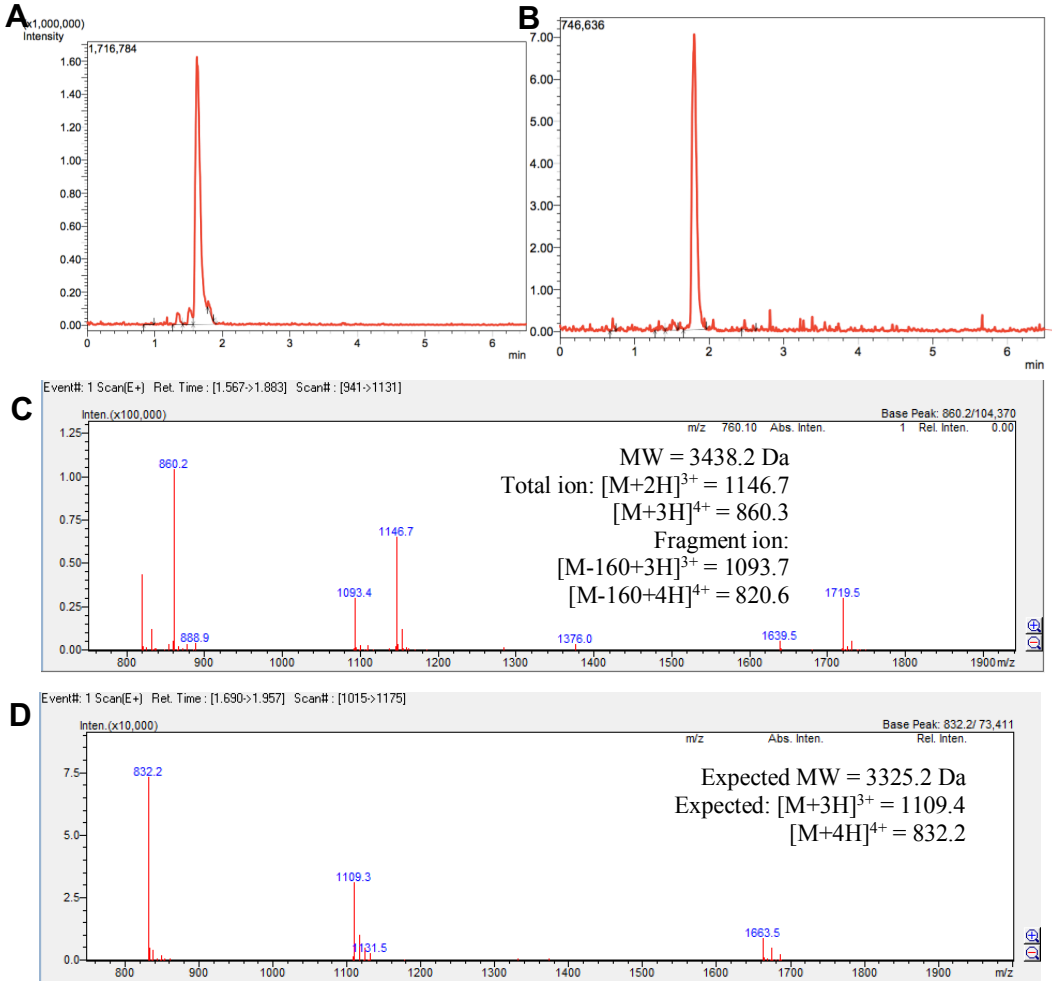
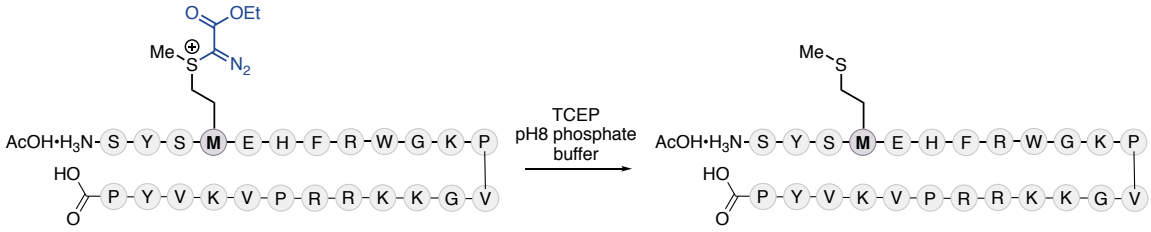


Figure S42: **(A)** Mass trace of **5**. **(B)** Mass trace of the crude mixture between **5** and TCEP at pH 8. **(C)** Mass spectrum of **5** (positive ionization mode, TIC from T_r=1.57-1.88 min.) **(D)** Mass spectrum of the crude mixture between **5** and TCEP at pH 8 (positive ionization mode, TIC from T_r=1.69-1.96 min.) showing full conversion to free aviptadil.

Phosphine promoted "De–ligation" of Tetracosactide conjugate 6



A vial was charged with a solution containing sulfonium conjugate **6** (650 µM, 15 µL). To the vial was added a solution of Tris-(2-carboxyethyl) phosphine (1.7 mM in pH 8 phosphate buffer, 50 µL) and H_2O (35 µL) and vial was allowed to stand for 30 minutes. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation reversal was judged to have proceeded in >95% conversion (**Figure S43**).

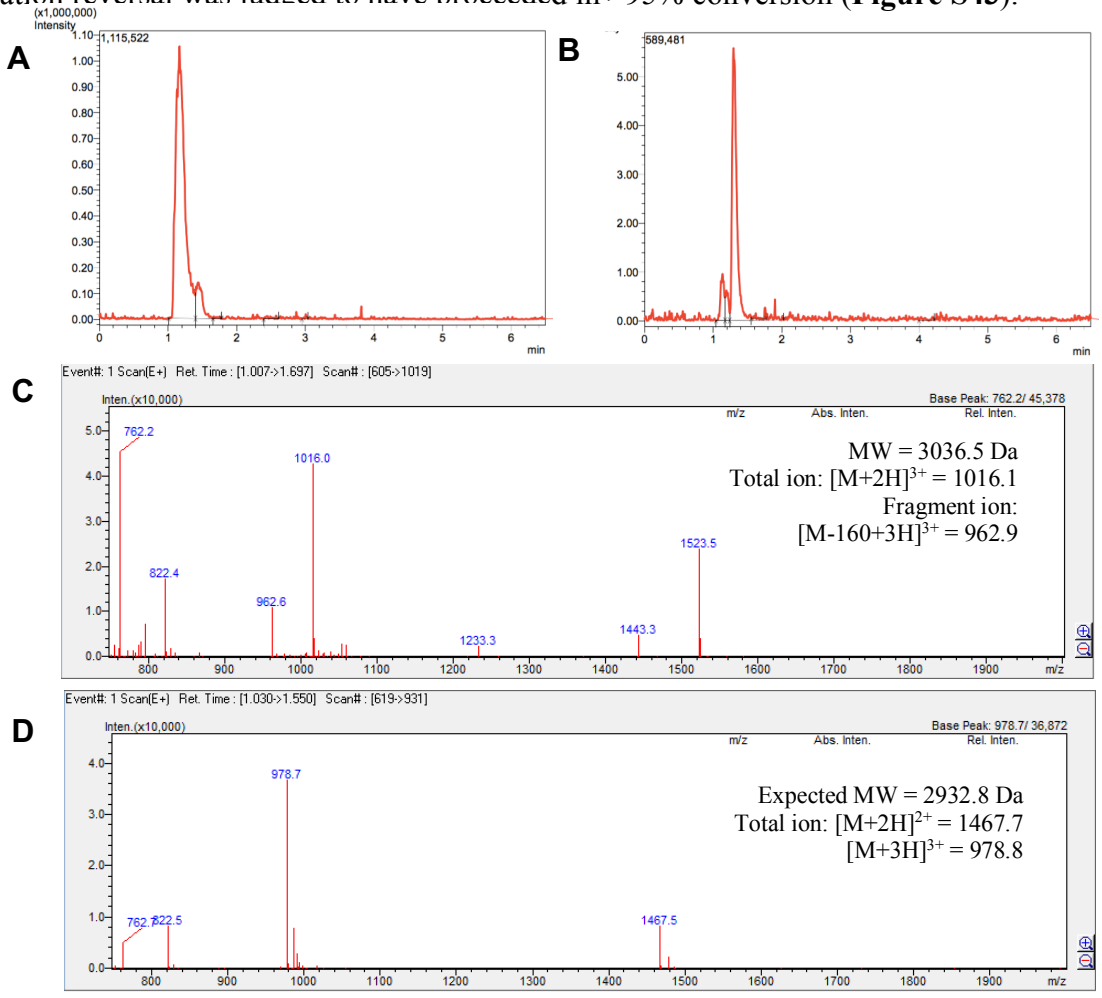
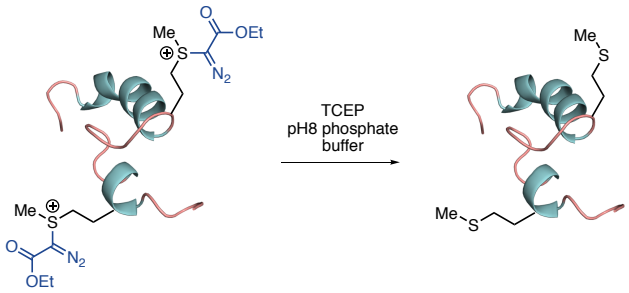


Figure S43: (**A**) Mass trace of **6**. (**B**) Mass trace of the crude mixture between **6** and TCEP at pH 8. (**C**) Mass spectrum of **6** (positive ionization mode, TIC from T_r=1.01-1.69 min.) (**D**) Mass spectrum of the crude mixture between **6** and TCEP at pH 8 (positive ionization mode, TIC from T_r=1.03-1.55 min.) showing full conversion to free tetracosactide.

Phosphine promoted "De–ligation" of Teriparatide conjugate 8



A vial was charged with a solution containing sulfonium conjugate **8** (500 µM, 20 µL). To the vial was added a solution of Tris-(2-carboxyethyl) phosphine (1.7 mM in pH 8 phosphate buffer, 50 µL) and H_2O (30 µL) and vial was allowed to stand for 30 minutes. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation reversal was judged to have proceeded in >95% conversion (**Figure**

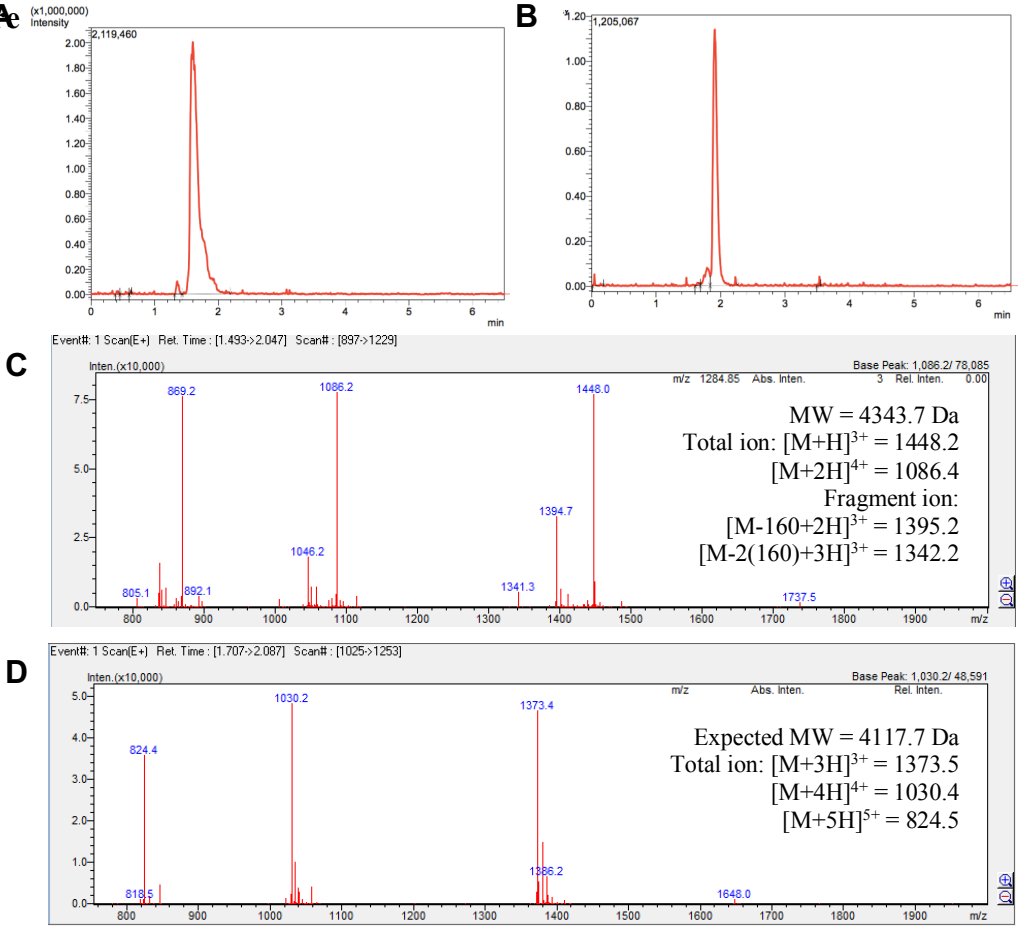
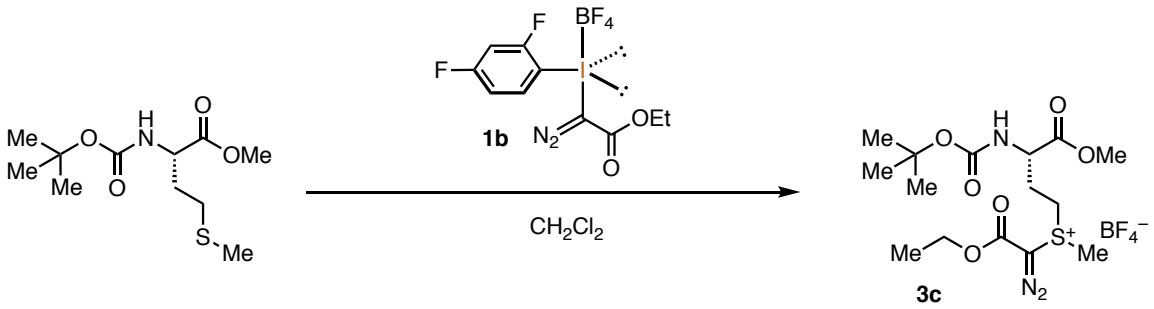


Figure S44: (**A)** Mass trace of **8**. **(B)** Mass trace of the crude mixture between **8** and TCEP at pH 8. **(C)** Mass spectrum of **8** (positive ionization mode, TIC from T_r=1.49-2.05 min.) **(D)** Mass spectrum of the crude mixture between **8** and TCEP at pH 8 (positive ionization mode, TIC from T_r=1.71-2.09 min.) showing full conversion to free teriparatide.

Photochemical Reduction of α-sulfoniumdiazoesters.

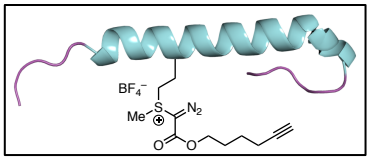
(6*S*)-10-diazo-6-(methoxycarbonyl)-2,2,9-trimethyl-4,11-dioxo-3,12-dioxa-9-thia-5-azatetradecan-9-ium tetrafluoroborate (3c).



A one-dram vial equipped with a magnetic stirrer was charged with methyl (*tert*-butoxycarbonyl)-*L*-methioninate (13 mg, 0.05 mmol). To the vial was added methylene chloride (0.5 mL). To the rapidly stirring solution was added **1b** (22 mg, 0.05 mmol) in a single portion, and the resulting solution was allowed to stir at room temperature for 1 hour. The magnetic stirrer was then removed, and the solution precipitated by addition of 40-60 petroleum ether and standing in a 4°C refrigerator. The supernatant was then decanted, and the residue dried *in vacuo* to yield **3c** as a faint yellow residue (22 mg, 93%, 1:1 mixture of diastereomers). **3c** was considerably hygroscopic and should be stored in a –20°C freezer.

^{1}H-NMR (500 MHz, CD_3CN) δ : 5.84 (s, br, 1H), 4.83 (app q, *J* = 7.1 Hz, 2H), 4.32 (s, br, 1H), 3.89-3.80 (m, 1H), 3.74 (s, 3H), 3.61-3.50 (m, 1H), 3.20 (s, 1.5 H), 3.19 (s, 1.5H), 2.45-2.36 (m, 1H), 2.23-2.10 (m, 1H), 1.45 (s, 9H), 1.37-1.32 (m, 3H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 171.1, 160.0 (2C), 155.8, 79.9, 79.8, 63.6, 53.7 (br), 52.2 (2C), 51.9 (br, 2C), 42.2 (br), 41.5 (br), 27.4, 26.6 (br), 26.5 (br), 13.3.
IR: 3389, 2979, 2146, 1700, 1516 (br), 1516, 1368, 1274, 1162, 854, 737.
HRMS-(ESI)$^+$ (m/z) Found (M^+): 376.1548, Calc'd $C_{15}H_{26}O_6N_3S$ requires 376.1537.

Functionalization of Exenatide•OAc with 1h to form 4g



The labeling of Exenatide•OAc was performed according to general procedure B using a solution of Exenatide•OAc (1 mM in 100 mM thiourea, 80 µL), Tempo (33 mM in H_2O, 120 µL), and **1h** (100 mM in CH_3CN) a reaction time of 30 minutes, and 1:1 diethyl ether:ethyl acetate in the extraction step. The resulting solution was then analyzed directly *via* LC/MS (Pentafluorophenyl column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >90% conversion (**Figure S45**).

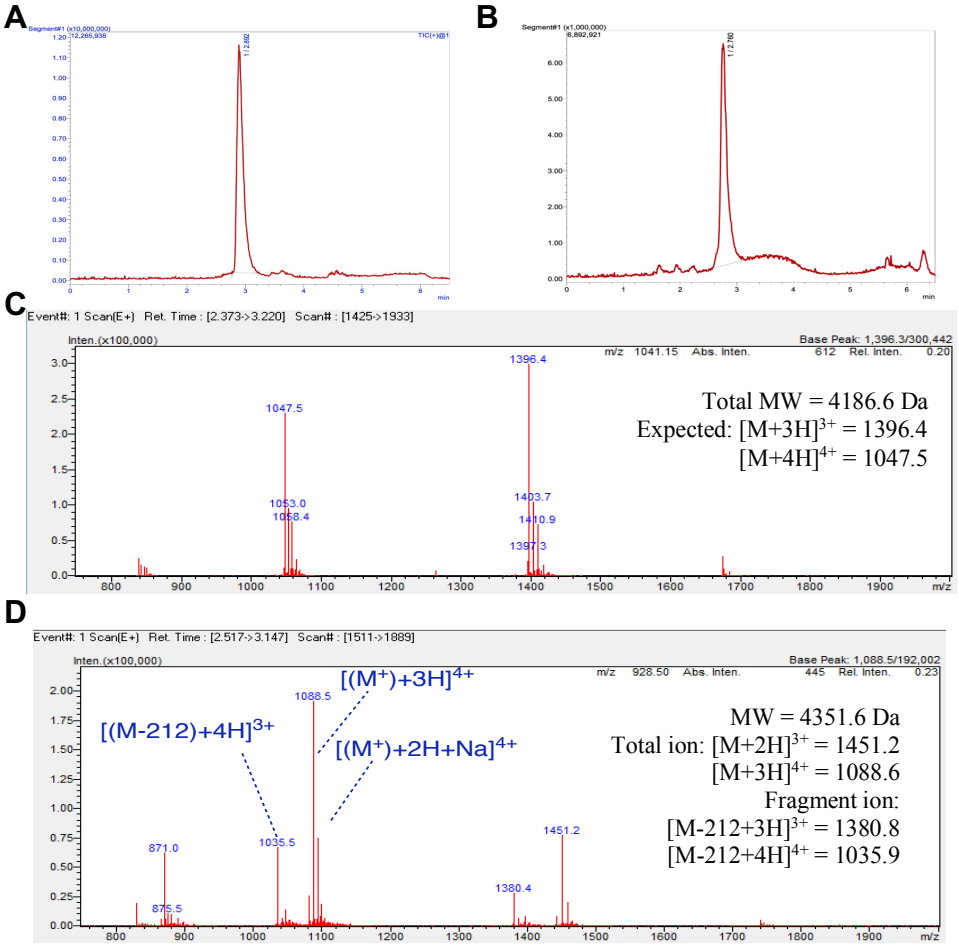
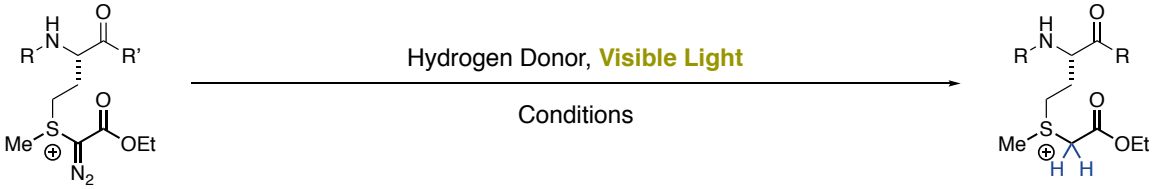


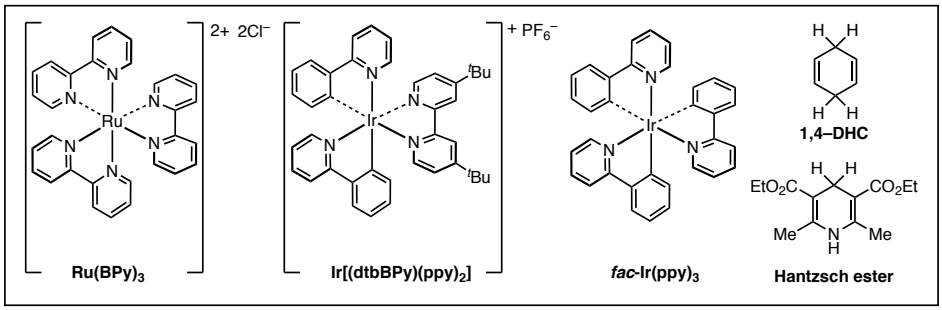
Figure S45. (**A**) Mass trace of parent Exenatide•OAc. **(B)** Mass trace of the crude mixture between Exenatide and **1h**. **(C)** Mass chromatogram of exenatide (positive ionization mode, TIC from T_r=2.5-3.2 min.) **(D)** Mass chromatogram of the crude reaction to form **4g** (positive ionization mode, TIC from T_r=2.53-3.15 min.)

Table S3. Optimization of the Photochemical Reduction Process.

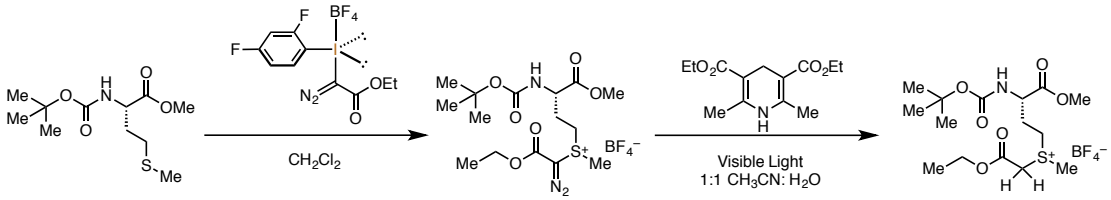


Entry	**3c** *or* **4g** Substrate	Photocatalyst ([cat])	Hydrogen Donor	Solvent	Atmosphere	%Conversion	**17a** *or* **4h** %17a or 4h
1	**3c** [5 mM]	$Ru(BPy)_3$ [1 mM]	1,4–CHD [10 mM]	CH_3CN	Sparged N_2	53%[a]	7%[a]
2	**3c** [5 mM]	$Ru(BPy)_3$ [1 mM]	none	CH_3CN	Sparged N_2	48%[a]	10%[a]
3	**3c** [5 mM]	$Ir[(dtbBPy)(ppy)_2]$ [1 mM]	1,4–CHD [10 mM]	CH_3CN	Sparged N_2	65%[a]	7%[a]
4	**3c** [5 mM]	$Ir[(dtbBPy)(ppy)_2]$ [1 mM]	none	CH_3CN	Sparged N_2	86%[a]	22%[a]
5	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	1,4–CHD [10 mM]	CH_3CN	Sparged N_2	81%[a]	19%[a]
6	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	none	CH_3CN	Sparged N_2	83%[a]	35%[a]
7	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	1,4–CHD [20 mM]	CH_3CN	Sparged N_2	83%[a]	36%[a]
8	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	1,4–CHD [40 mM]	CH_3CN	Sparged N_2	98%[a]	43%[a]
9	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	1,4–CHD [40 mM]	$(CH_3)_2CO$	Sparged N_2	92%[a]	54%[a]
10	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [1 mM]	1,4–CHD [40 mM]	7:3 $(CH_3)_2CO$:H_2O	Sparged N_2	61%[a]	32%[a]
11	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [500 μM]	Hantzsch Ester [15 mM]	1:1 $(CH_3)_2CO$:H_2O	Sparged N_2	>95%[b]	94%[b]
12	**3c** [5 mM]	*fac*-$Ir(ppy)_3$ [500 μM]	Hantzsch Ester [15 mM]	1:1 CH_3CN:H_2O	Sparged N_2	>95%[b]	95%[b]
13	**3c** [5 mM]	none	Hantzsch Ester [15 mM]	1:1 CH_3CN:H_2O	Sparged N_2	>95%[b]	93%[b]
14[c]	**3c** [5 mM]	none	Hantzsch Ester [20 mM]	1:1 CH_3CN:H_2O	Sparged N_2	20%[b]	trace[b]
15	**4g**[d] [200 μM]	none	Hantzsch Ester [20 mM]	1:1 CH_3CN:H_2O	Sparged N_2	<10%[a]	<10%[a]
16	**4g**[d] [200 μM]	*fac*-$Ir(ppy)_3$ [200 μM]	Hantzsch Ester [20 mM]	1:1 CH_3CN:H_2O	Sparged N_2	90%[a]	90%[a]

[a]Yield estimated from TIC *via* LCMS. [b]Yield estimated *via* ^{1}H-NMR using 1,1,2,2-tetrachloroethane as an internal standard.
[c]Reaction run in the dark using deuterated solvents. [d]**4g** was used directly from the conjugation reaction without purification.



6-(methoxycarbonyl)-2,2,9-trimethyl-4,11-dioxo-3,12-dioxa-9-thia-5-azatetradecan-9-ium tetrafluoroborate (17a)

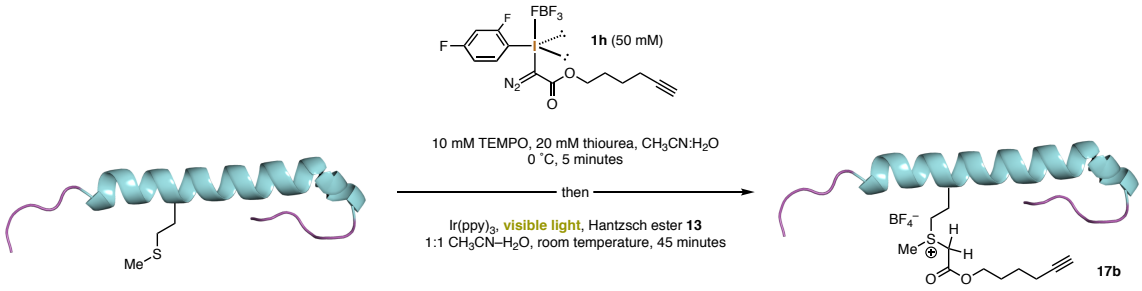


A vial equipped with a magnetic stirrer was charged with methyl (*tert*-butoxycarbonyl)-*L*-methioninate (13 mg, 0.05 mmol). To the vial was added methylene chloride (0.5 mL). To the rapidly stirring solution was added **1b** (22 mg, 0.05 mmol) in a single portion, and the resulting solution was allowed to stir at room temperature for 1 hour. The magnetic stirrer was then removed, and the solvent was removed using a rotary evaporator. The resulting residue was then triturated 5 times with diethyl ether using a sonicator. The vial was then evacuated and refilled with N_2, and 2.5 mL of freshly sparged CH_3CN was added.

A separate microwave tube equipped with a magnetic stirrer was charged with Hantzsch ester (25 mg, 0.098 mmol), capped with a rubber septum, and then evacuated and refilled with N_2. To the tube was added freshly sparged water (2.5 mL) and the CH_3CN solution containing **3c** (2.5 mL) sequentially. The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 2 hours. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then transferred to a round-bottomed flask and the solvent was removed using a rotary evaporator. The resulting residue was then triturated 3 times with diethyl ether in order to remove the excess Hantzsch ester/byproducts and the supernatant carefully decanted. The remaining residue was then dried *in vacuo* to yield **17a** as an off-white, amorphous residue (18 mg, 82%, 1:1 mixture of diastereomers).

^{1}H-NMR (400 MHz, CD_3CN): 5.82 (s, br, 1H), 4.35-4.24 (m, 5H), 3.74 (s, 3H), 3.52-3.44 (m, 1H), 3.41-3.34 (m, 1H), 2.94 (s, 1.5H), 2.94 (s, 1.5H), 2.44-2.43 (m, 1H), 2.23-2.09 (m, 1H), 1.45 (s, 9H), 1.32 (app t, J = 7.2 Hz, 3H).
^{13}C-NMR (125 MHz, CD_3CN): 172.1, 164.8 (2C), 156.7 (br), 80.7, 64.5, 53.1, 53.0 (br), 44.7, 44.5, 39.9, 39.6, 28.4 (2C), 27.1, 27.0, 24.2 (2C), 14.1.
IR: 2992, 1731 (br), 1513, 1316, 1028 (br), 780.
HRMS-$(ESI)^+$ (m/z) Found (M^+): 350.1643, Calc'd $C_{15}H_{18}O_6NS$ requires 350.1632.

Two-step sequential Methionine-Labelling/photochemical Reduction of Exenatide•OAc (17b)



A 5 mL microwave tube equipped with a magnetic stirrer was charged with a solution of exenatide•OAc (3.7 mM in 0.1 M thiourea (aq), 70 µL, 1 mg). To the vial was added formic acid (100 mM in H_2O, 20 µL), and the resulting solution chilled in an ice bath. To the solution was added TEMPO (33 mM in H_2O, 50 µL), H_2O (30 µL) and the solution was stirred rapidly. To the rapidly stirring solution was immediately added **1h** (100 mM in CH_3CN, 170 µL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (2.6 mg, $1.0x10^{-5}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-$Ir(ppy)_3$ (560 µM in freshly sparged CH_3CN, 200 µL) and freshly sparged H_2O (170 µl). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 45 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation was judged to have proceeded in 90% conversion. Purification *via* reverse-phase HPLC (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95%B over 1min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **4h** as a free-flowing, white solid that was immediately re-dissolved in H_2O (0.5 mL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.8 mg/mL solution (90% isolated yield). **4h** contains exenatide sulfoxide as a minor impurity (<5%).

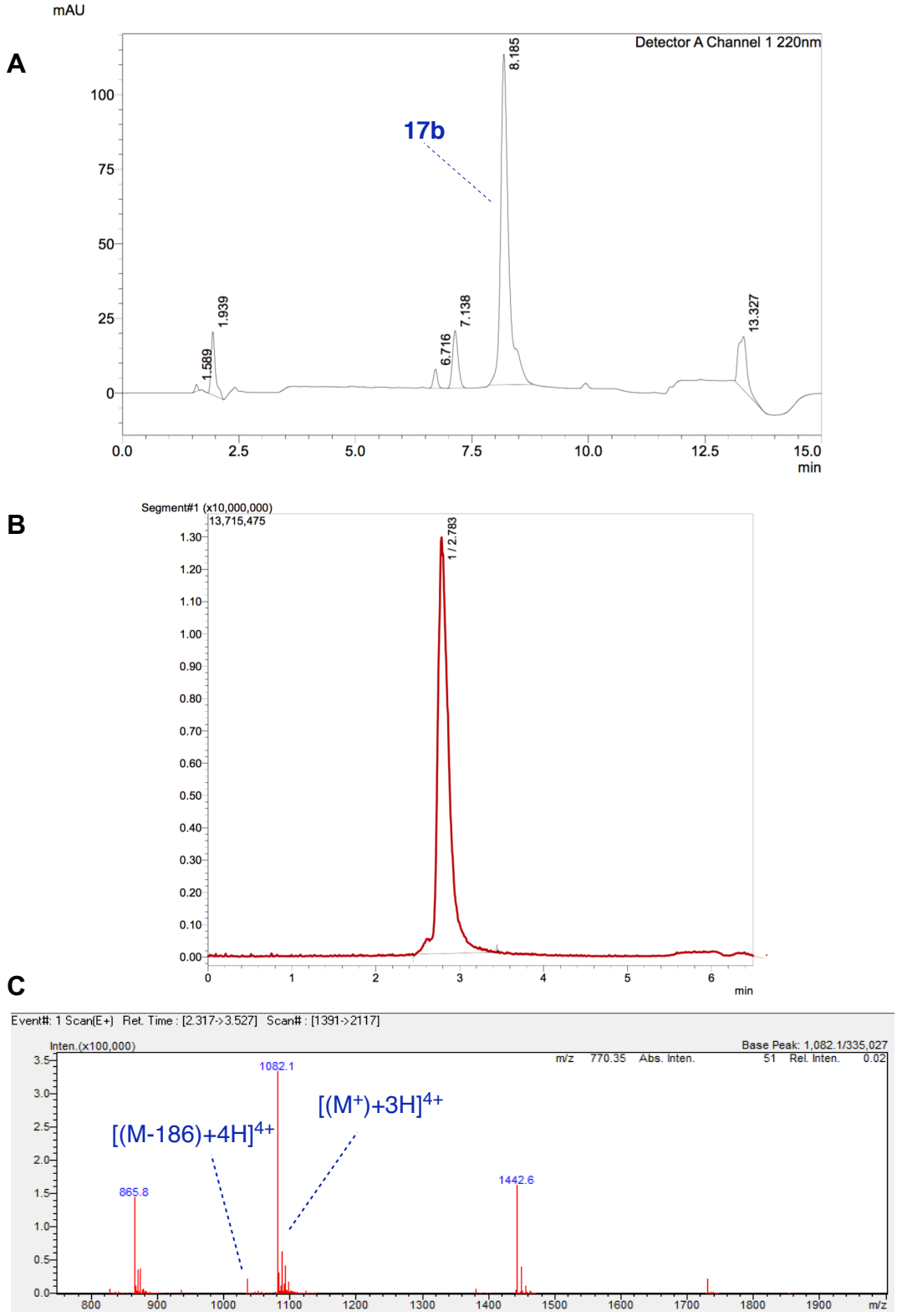
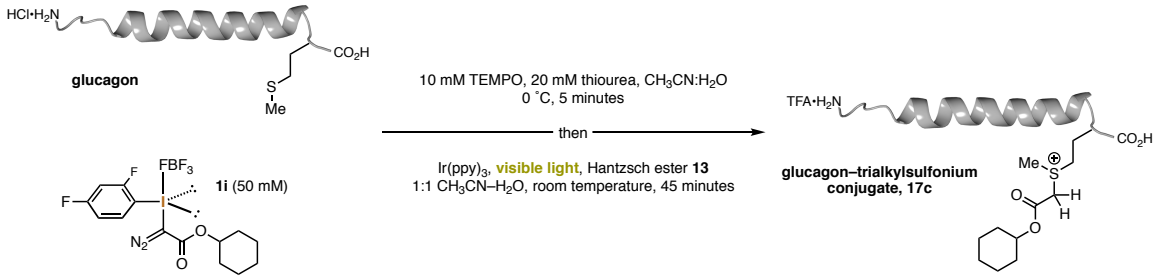


Figure S46. (A) HPLC trace of the crude reaction mixture to form **4h**. **(B)** Mass trace of purified **17b**. **(C)** Mass chromatogram of purified **17b**.

Two-step sequential Methionine Labeling/photochemical Reduction of Glucagon•HCl with iodonium salt 1i (17c)



A 5 mL microwave tube equipped with a magnetic stirrer was charged with a solution of glucagon•HCl (3.3 mM in 0.1 M thiourea (aq), 70 µL, 0.8 mg). To the vial was added formic acid (100 mM in H_2O, 18 µL), and the resulting solution chilled in an ice bath. To the solution was added TEMPO (33 mM in H_2O, 90 µL), and the solution was stirred rapidly. To the rapidly stirring solution was immediately added **1h** (0.1 M in CH_3CN, 190 µL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (2 mg, $0.7x10^{-3}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-Ir(ppy)$_3$ (560 µM in freshly sparged CH_3CN, 140 µL) and freshly sparged acetonitrile (35 µl). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 45 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation was judged to have proceeded in >95% conversion. Purification *via* reverse-phase HPLC (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95%B over 1 min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **17c** as a free-flowing, white solid that was immediately re-dissolved in H_2O (1 mL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.3 mg/mL solution (90% isolated yield).

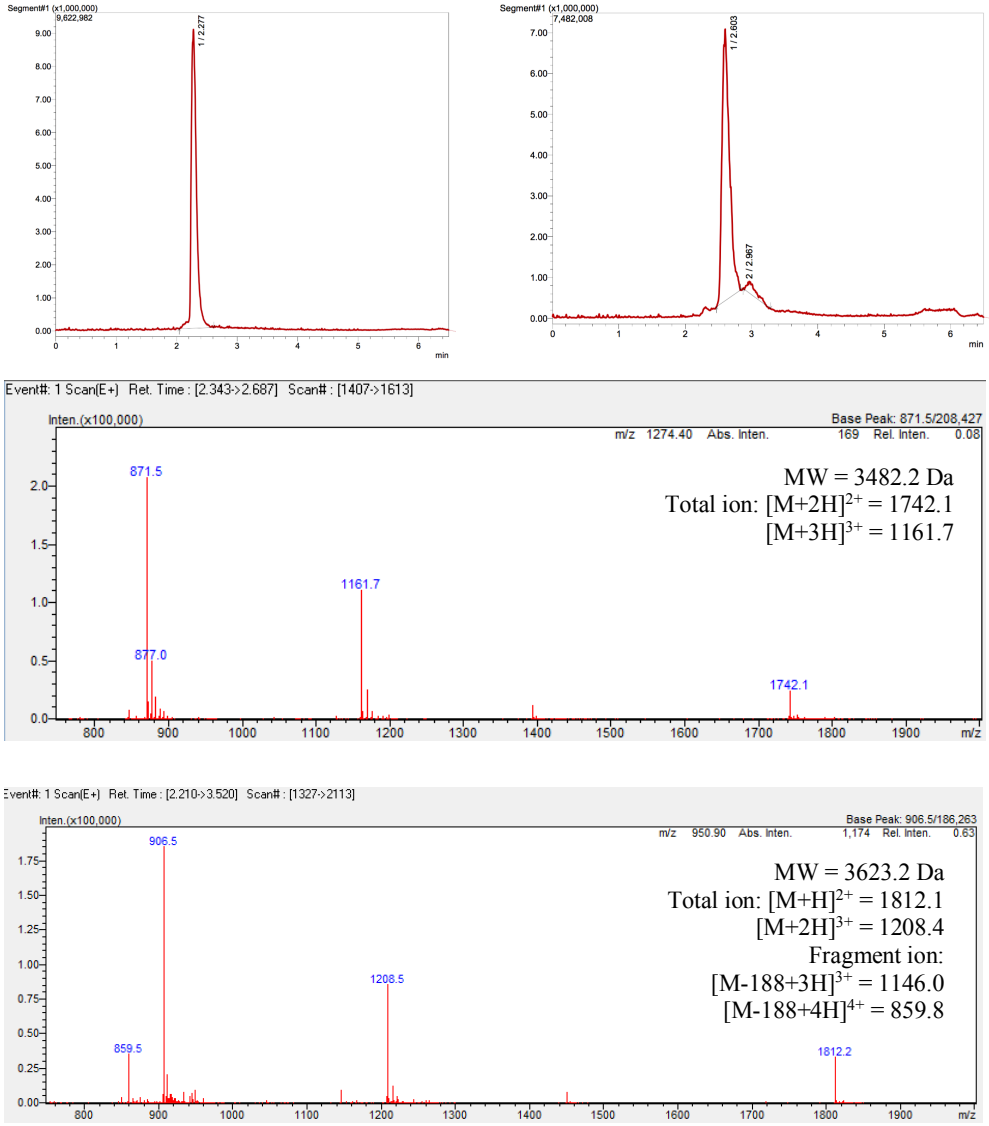


Figure S47. (**A)** Mass trace of parent Glucagon•HCl. **(B)** Mass trace of crude reaction mixture of the labeling/photoreduction of Glucagon with **1i** to form **17c**. **(C)** Mass spectrum of Glucagon (positive ionization mode, TIC from T_r=2.34-2.69 min.) **(D)** Mass spectrum of the crude reaction to form **17c** (positive ionization mode, TIC from T_r=2.21-3.52 min.)

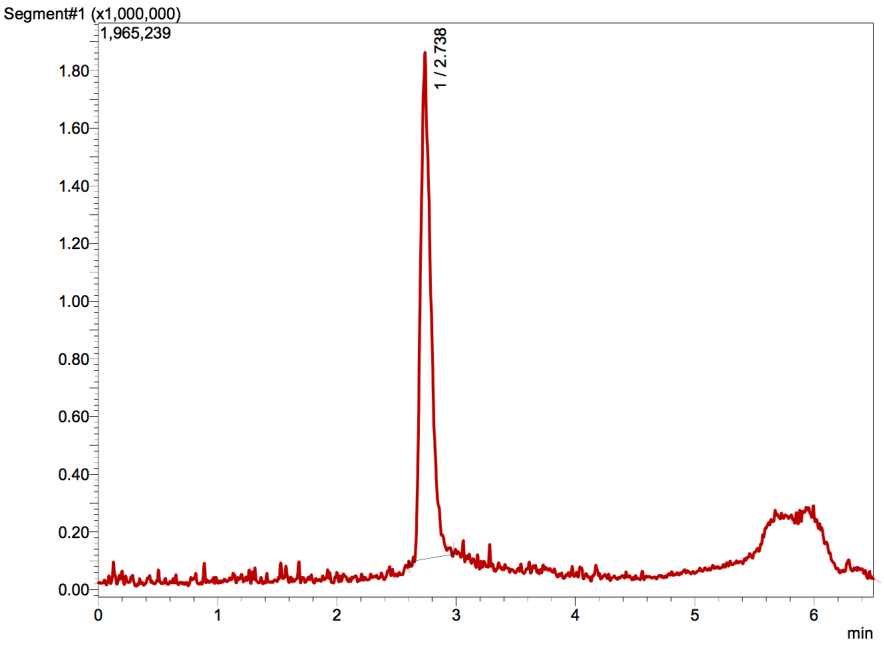
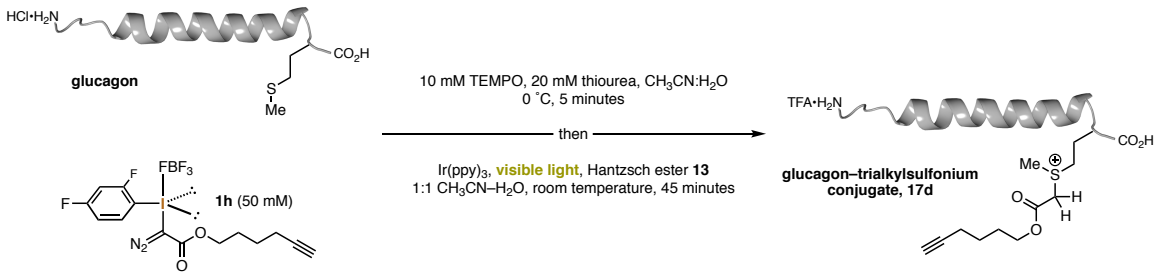


Figure S48. (**A)** Mass-trace of **17c** after purification by HPLC.

Two-step sequential Methionine Labeling/photochemical Reduction of Glucagon•HCl (17e)



A 5 mL microwave tube equipped with a magnetic stirrer was charged with a solution of glucagon•HCl (3.3 mM in 0.1 M thiourea (aq), 140 µL, 1.6 mg). To the vial was added formic acid (100 mM in H_2O, 35 µL), and the resulting solution chilled in an ice bath. To the solution was added TEMPO (33 mM in H_2O, 175 µL), and the solution was stirred rapidly. To the rapidly stirring solution was immediately added **1h** (0.1 M in CH_3CN, 350 µL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (4 mg, $1.4x10^{-2}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-Ir(ppy)$_3$ (560 µM in freshly sparged CH_3CN, 280 µL) and freshly sparged acetonitrile (70 µl). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 45 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation was judged to have proceeded in >95% conversion. Purification *via* reverse-phase HPLC (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95%B over 1min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **17e** as a free-flowing, white solid that was immediately redissolved in H_2O (1 mL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.3 mg/mL solution (80% isolated yield). A similar reaction was performed at a lower concentration of peptide using glucagon•HCl (1 mM in 0.1 thiourea(aq), 40 µL), TEMPO (33 mM in H_2O, 40 µL), CH_3CN (20 µL) and **1h** (100 mM in CH_3CN, 100 µL) for the labeling reaction; and Hantzsch ester (2 mg), freshly sparged H_2O (120 µL) and *fac*-Ir(ppy)$_3$ (800 µM in freshly sparged CH_3CN, 200 µL) for the photoreduction step. The two-step process was found to have proceeded in >95% conversion by LC/MS. ICP-MS of the solution containing isolated conjugate 17e indicated trace Ir levels of less than 15 ng/mL.

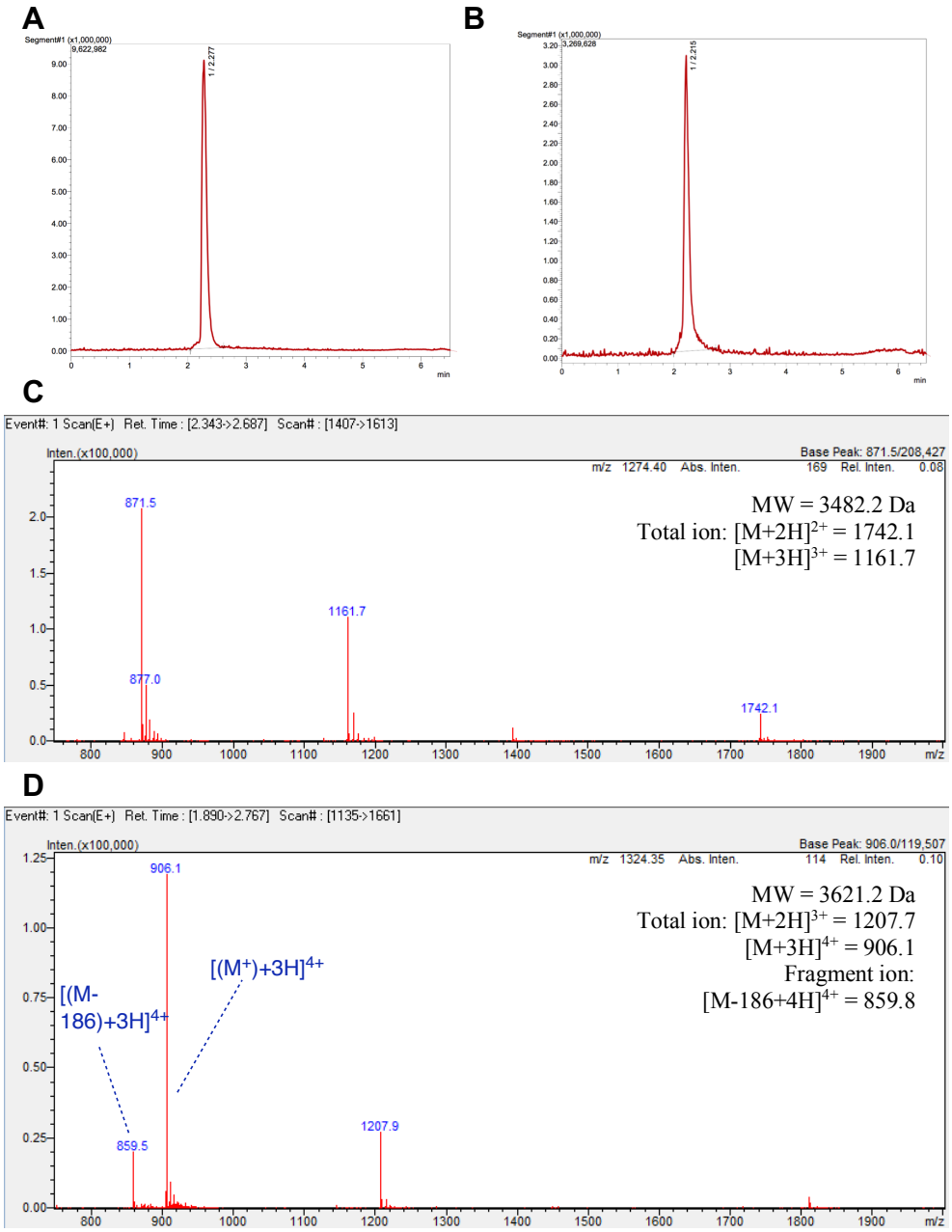


Figure S49. (**A)** Mass trace of parent Glucagon•HCl. **(B)** Mass trace of crude reaction mixture of the labeling/photoreduction of Glucagon with **1h** to form **17e**. **(C)** Mass spectrum of Glucagon (positive ionization mode, TIC from T_r=2.34-2.69 min.) **(D)** Mass spectrum of the crude reaction to form **17e** (positive ionization mode, TIC from T_r=1.89-2.77 min.)

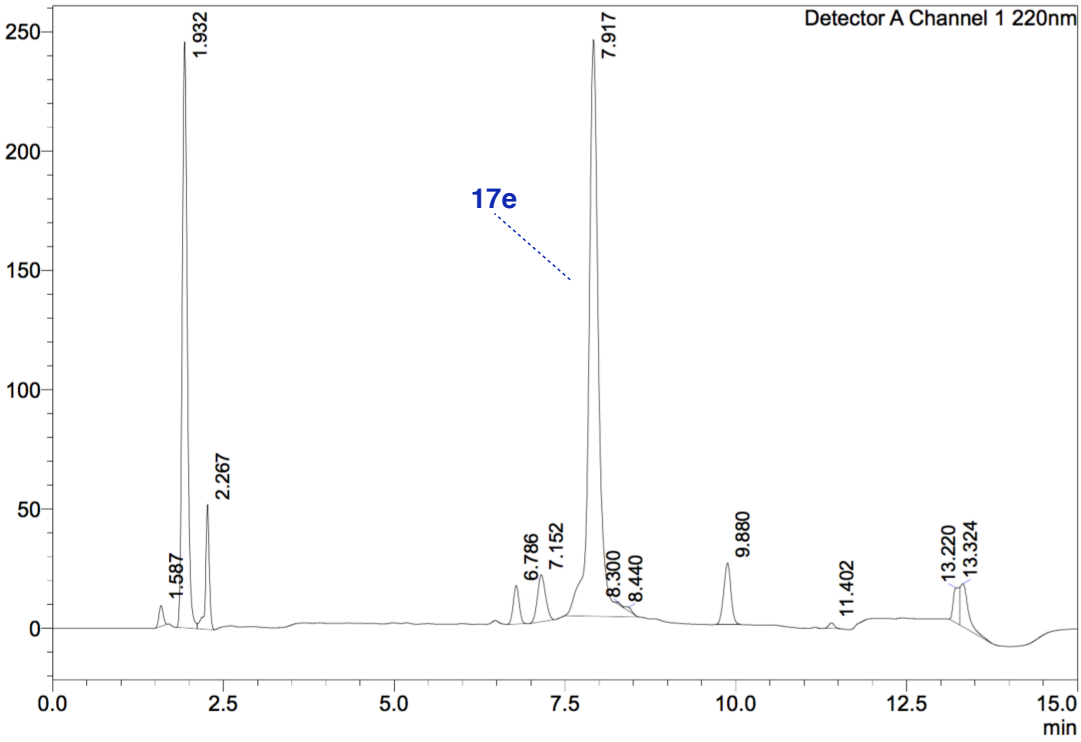


Figure S50. HPLC trace of the crude reaction mixture to form **17e**.

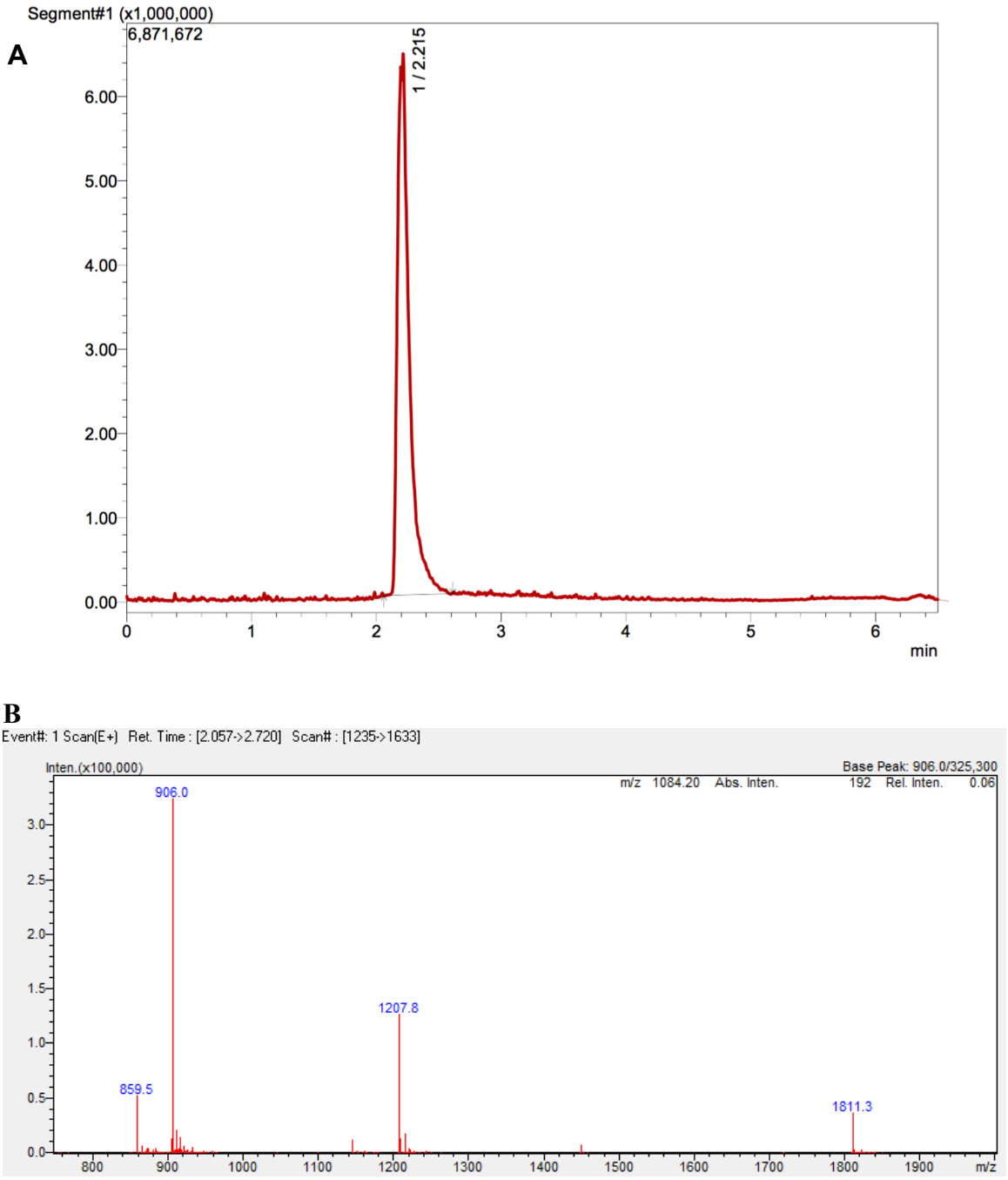


Figure S51. (A) HPLC trace Purified **17e. B)** Mass spectrum of purified **17e** (TIC from 2.06-2.72 min)**.**

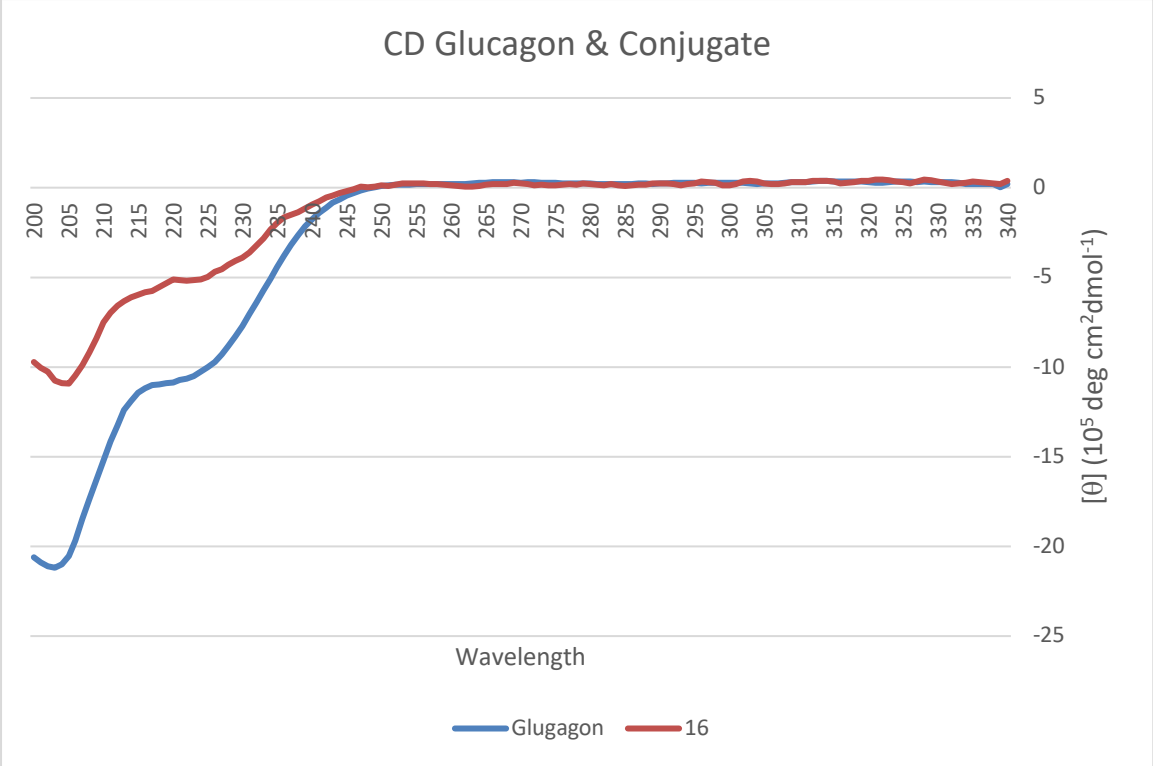
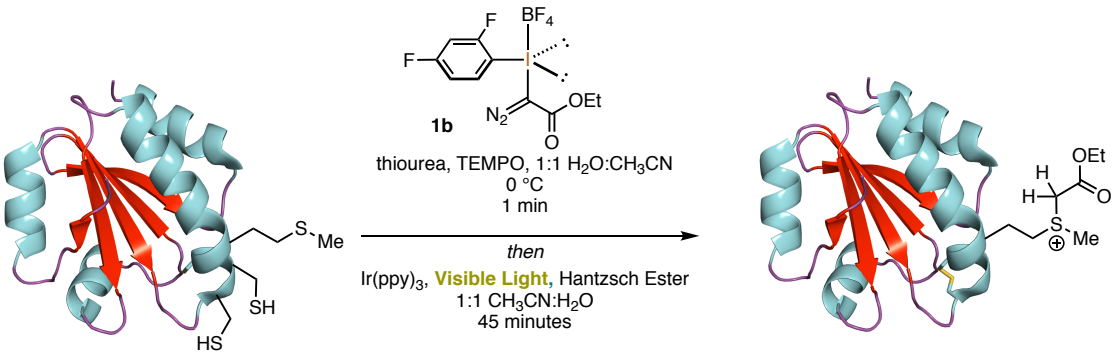


Figure S52. CD spectra of Glucagon (blue line) and **17e** (red line) at 50 μM in pH 7.4 phosphate buffer. **17e** retains the structural characteristics of parent Glucagon.

Two-step sequential Methionine-Labelling/photochemical Reduction of Thioredoxin (17d)



A 2 mL vial equipped with a magnetic stirrer was evacuated and refilled with N_2. To the vial was added an N_2-sparged solution containing Thioredoxin (170 µM in 0.1 M thiourea (aq), 20 µL), formic acid (100 mM, 10 µL), and N_2-sparged H_2O (50 µL). The resulting solution was chilled in an ice bath. To the chilled solution was added an N_2-sparged solution containing TEMPO (33 mM in H_2O, 30 µL), and the resulting solution was stirred vigorously. To the stirring solution was added **1b** (100 mM in CH_3CN, 50 µL) and the resulting solution stirred for 1 minute. The resulting mixture was then extracted twice with 1:1 diethyl ether:ethyl acetate. The remaining organic volatiles were then removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (1 mg, $4x10^{-3}$ mmol). The vial was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the vial was added added *fac*-Ir(ppy)$_3$ (560 µM in freshly sparged CH_3CN, 80 µL). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 45 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted 3 times with ethyl acetate and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS, (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min), and was judged to have proceeded in 95% conversion with a (8:1.2:1) label:label+oxidation:double label ratio (**Figure S53**).

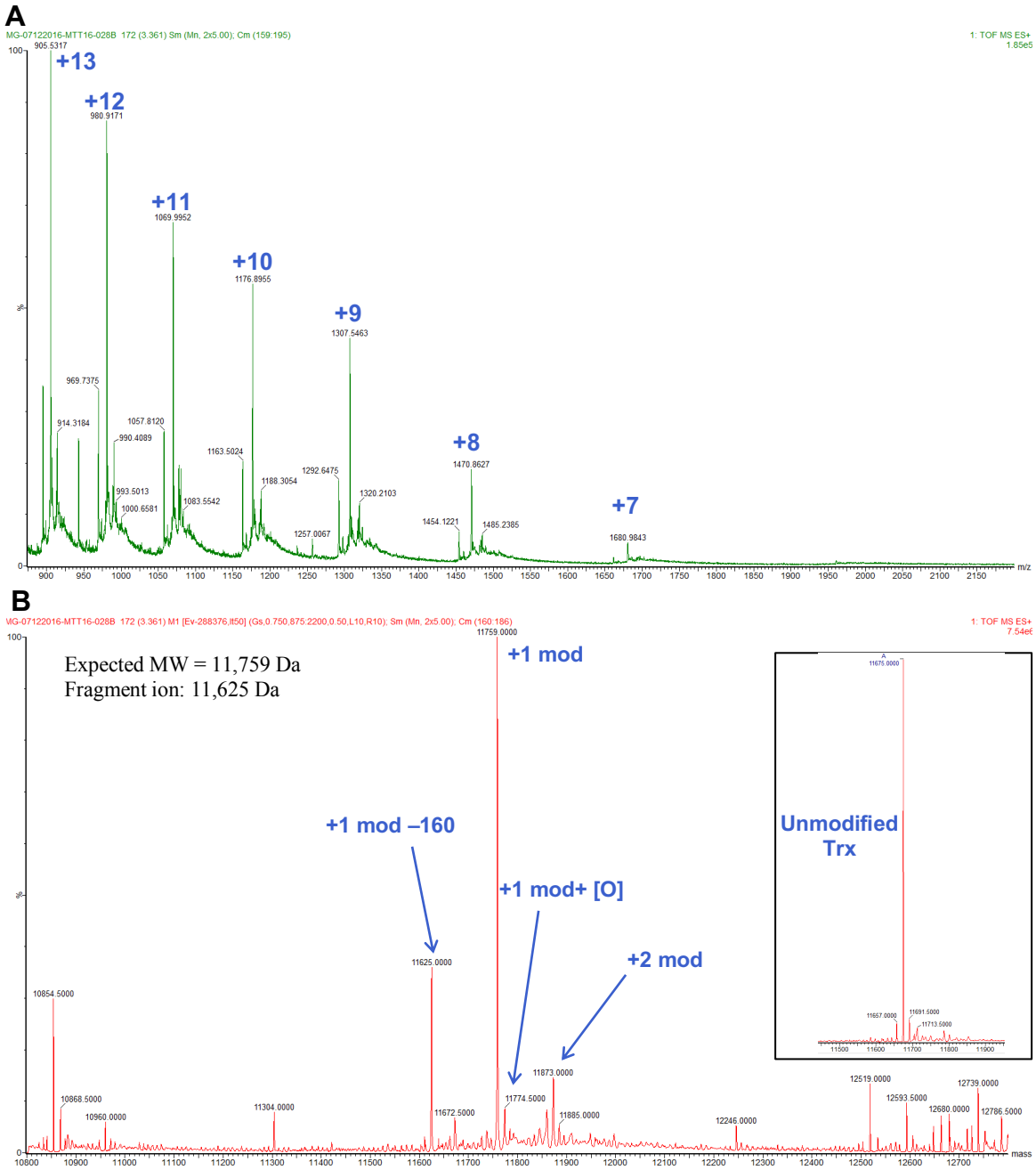
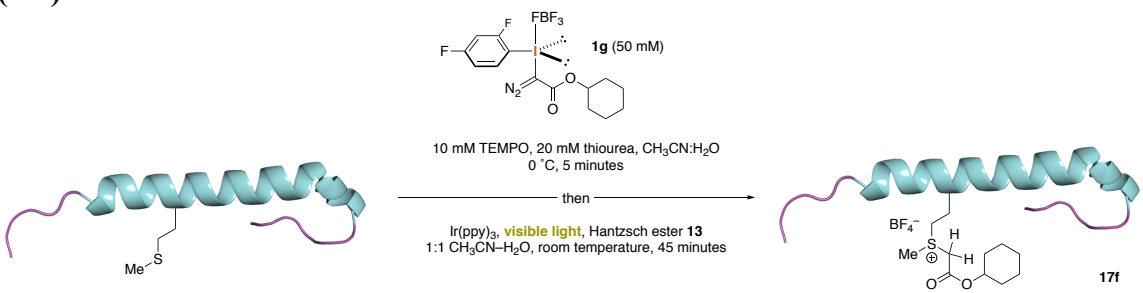


Figure S53: **(A)** Mass spectrum of the crude reaction mixture of the labeling/photoreduction of thioredoxin with **1b** to form **17d**. **(B)** Deconvoluted mass spectrum of the crude reaction to form **17d**. The insert depicts the deconvoluted mass spectrum of unmodified, reduced, thioredoxin.

Two-step sequential Methionine-Labelling/photochemical Reduction of Exenatide (17f)



A 5 mL microwave tube equipped with a magnetic stirrer was charged with a solution of exenatide•OAc (3.7 mM in 0.1 M thiourea (aq), 70 µL, 1 mg). To the vial was added formic acid (100 mM in H_2O, 20 µL), and the resulting solution chilled in an ice bath. To the solution was added TEMPO (33 mM in H_2O, 50 µL), H_2O (30 µL) and the solution was stirred rapidly. To the rapidly stirring solution was immediately added **1g** (100 mM in CH_3CN, 170 µL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (2.6 mg, $1.0x10^{-5}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-$Ir(ppy)_3$ (560 µM in freshly sparged CH_3CN, 200 µL) and freshly sparged H_2O (170 µl). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 45 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation was judged to have proceeded in >95% conversion. Purification *via* reverse-phase HPLC (C18 column (150 x 10 mm, 10µm), Gradient: 5-55% B over 9 min then 55-95%B over 1min, hold for 1.5 min, then 95-5%B over 0.5 min, hold for 3 min, 5 ml/min), followed by lyophilization yielded **17f** as a free-flowing, white solid that was immediately re-dissolved in H_2O (0.5 mL) and stored at –20°C. Concentration analysis by A_{280} indicated a 1.0 mg/mL solution (50% isolated yield).

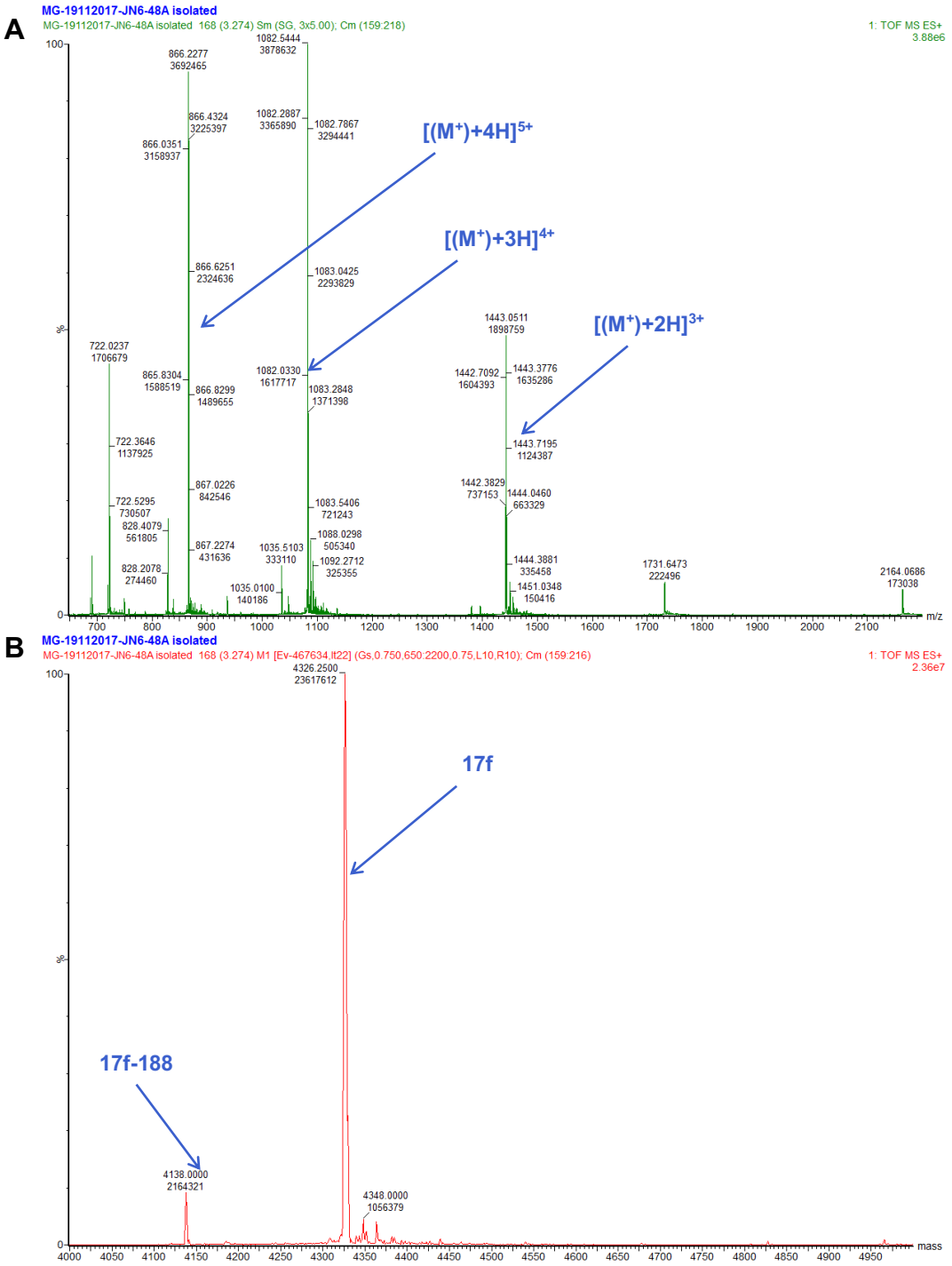
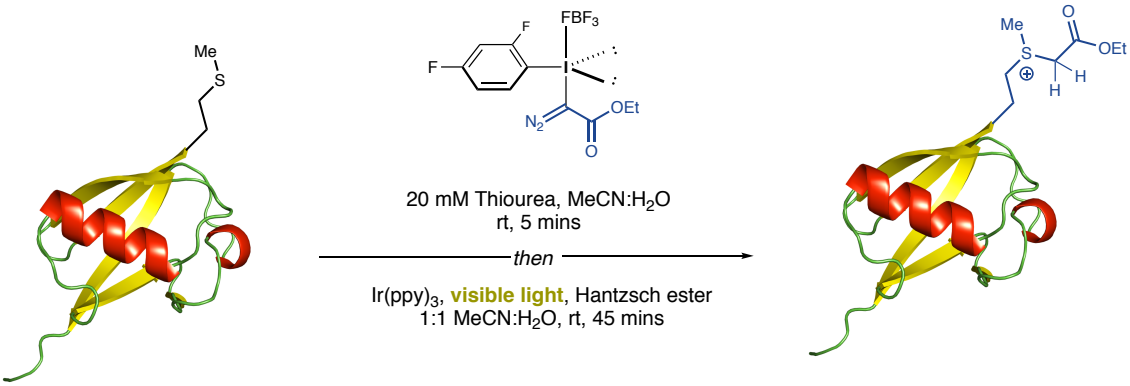


Figure S54. (**A)** Mass spectrum of the crude reaction mixture of the labeling/photoreduction of exenatide with **1g** to form **17f**. **(B)** Deconvoluted mass spectrum of the crude reaction to form **17f**.

Two-step sequential Methionine-Labelling/photochemical Reduction of Ubiquitin (17g)



A 2 mL vial equipped with a magnetic stirrer was charged with Ubiquitin (~0.5 mg, ~0.06 µmol) and evacuated and refilled with N_2 3 times. To the vial was added formic acid (100 mM in H_2O, 10 µL) and thiourea (0.1 M in H_2O, 40 µL) and H_2O (50 µL). To the rapidly stirring solution was immediately added **1g** (100 mM in CH_3CN, 100 µL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (2.0 mg, $1.0x10^{-5}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-$Ir(ppy)_3$ (560 µM in freshly sparged CH_3CN, 200 µL). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 30 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LCMS, (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min) and was judged to have proceeded in approximately 72% conversion over two steps (**Figure S55**).

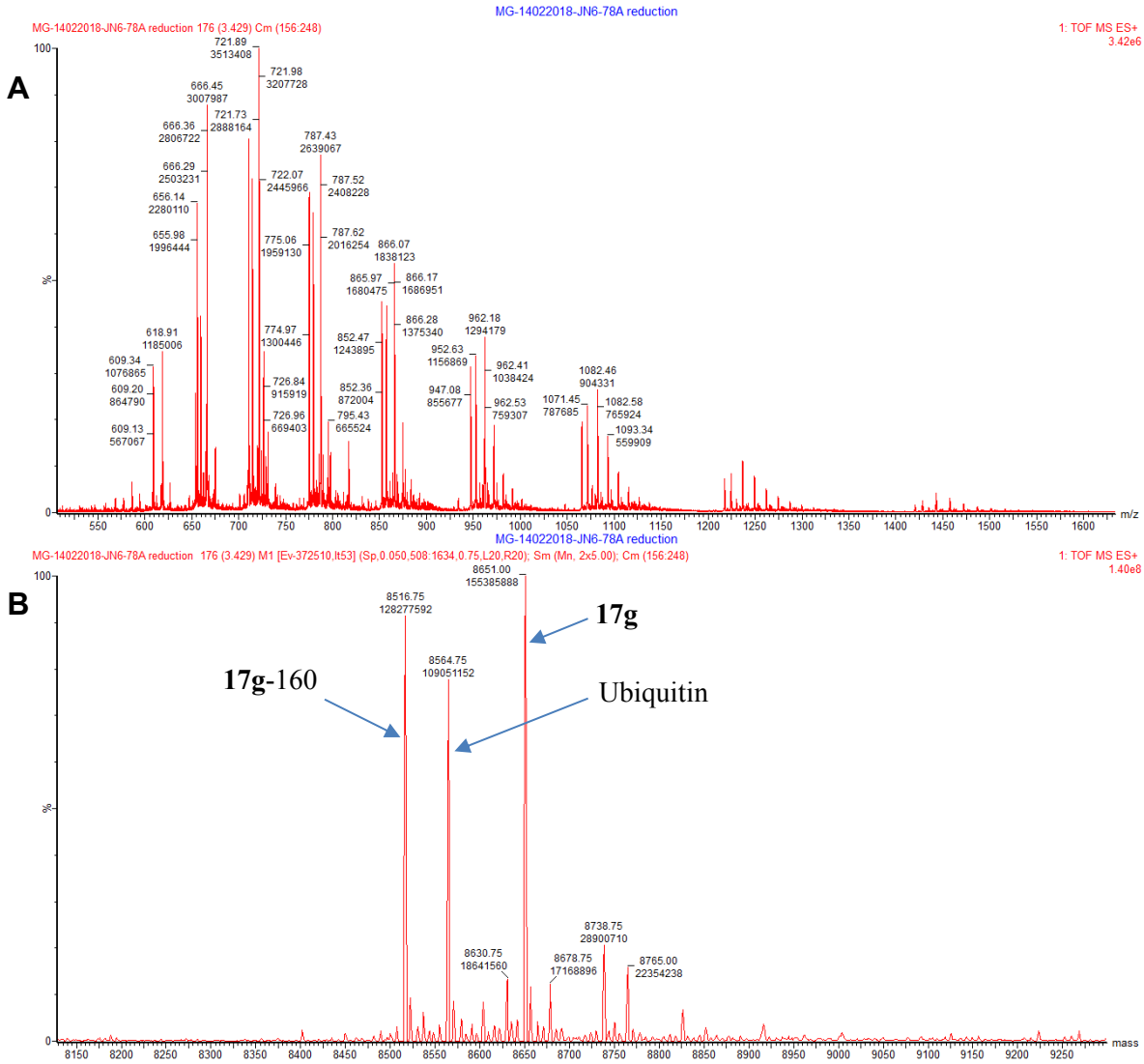


Figure S55: (**A**) Mass trace of crude two-step reaction of Ubiquitin to form **17g**. (**B**) Deconvoluted mass spectrum of the crude reaction to form **17g**.

Large scale two-step sequential Methionine-Labelling/photochemical Reduction of Ubiquitin for isolation (17g)

This larger scale reaction was run to partial completion in order to exemplify the purification of starting Ubiquitin, diazoester conjugate **11 and trialkylsulfonium conjugate **17g**.*

A 2 mL vial equipped with a magnetic stirrer was charged with Ubiquitin (~1.0 mg, ~0.12 μmol) and evacuated and refilled with N_2 3 times. To the vial was added formic acid (100 mM in H_2O, 25 μL) and thiourea (0.1 M in H_2O, 100 μL) and H_2O (125 μL). To the rapidly stirring solution was immediately added **1g** (100 mM in CH_3CN, 250 μL), and the resulting solution was allowed to stir for 5 minutes. The reaction mixture was then extracted 3 times with 1:1 ethyl acetate: diethyl ether, and the excess volatiles were removed from the aqueous layer using a rotary evaporator. To the resulting solution was added a magnetic stirrer and the Hantzsch ester (2.0 mg, $1.0x10^{-5}$ mmol). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added *fac*-$Ir(ppy)_3$ (560 μM in freshly sparged CH_3CN, 200 μL). The resulting heterogeneous mixture was then stirred rapidly. The mixture was irradiated with a Prolite 30-watt fluorescent light bulb that was placed at a 5 centimeter distance from the microwave tube for 30 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted three times with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then purified directly using MonoS 4.6/100 PE column (GE Healthcare) using a NaCl gradient (0-1 M) in 50 mM ammonium acetate pH 4.5 (**Figure S56**).

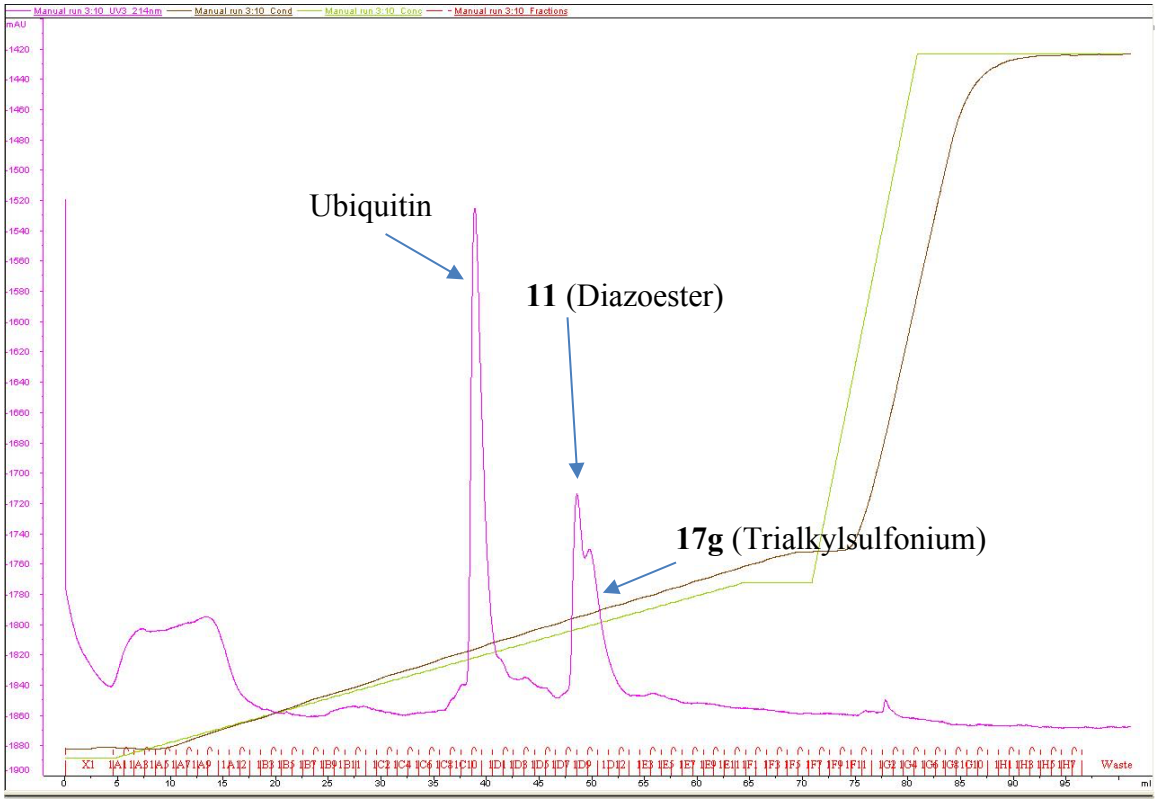


Figure S56: FPLC trace of Ubiquitin conjugate **17g**, purified using MonoS 4.6/100 PE column (GE Healthcare) using a NaCl gradient (0-1 M) in 50 mM ammonium acetate pH 4.5. Clear separation of unmodified Ubiquitin and modified conjugate **17g** was achieved due to the differing elution times of the charged species **17g** and uncharged, unmodified ubiquitin using an ion exchange column. Diagnostic fragment ion of [Ub+mod-160] MW = 8516.5 Da was observed in the product peak of **17g**. Hence, this indicates that the diagnostic fragmentation must be an artefact of the mass spectrometer, as proposed, otherwise the uncharged resulting species would have a dramatically different retention time using this ion exchange purification method.

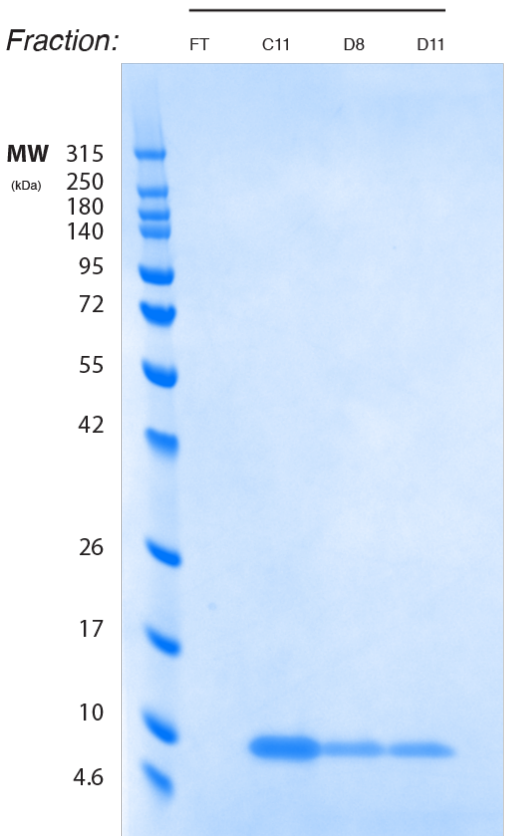
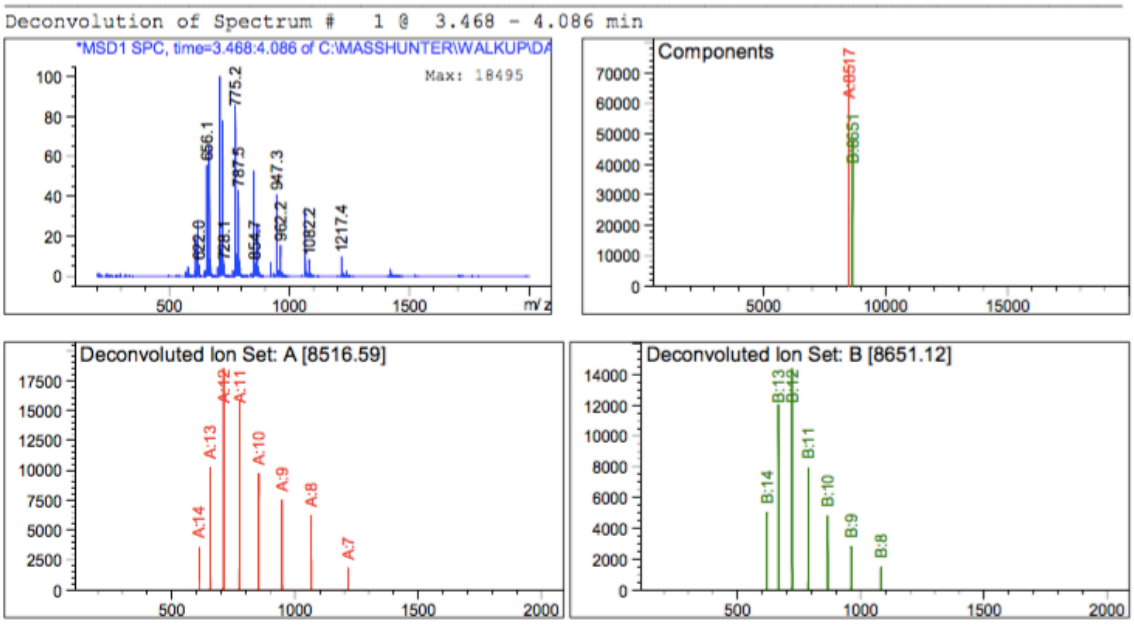
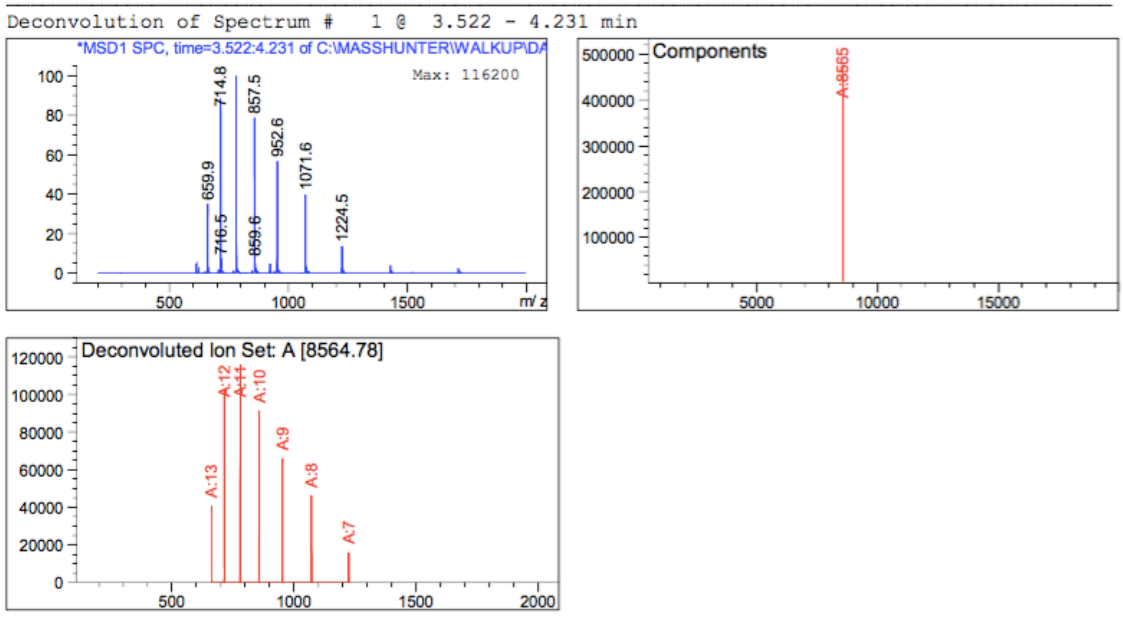


Figure S57: Gel of purified Ubiquitin fractions from FPLC purification run with Bolt 4-12% Bis-Tris gels (Thermo Scientific) and were run for 30 min at 165V (constant) using MES buffer (Thermo Scientific). The ladder on the gel is ProSieve QuadColor protein marker (4.6 kDa – 300 kDa; Lonza) and the gel was stained using InstantBlue (Generon)



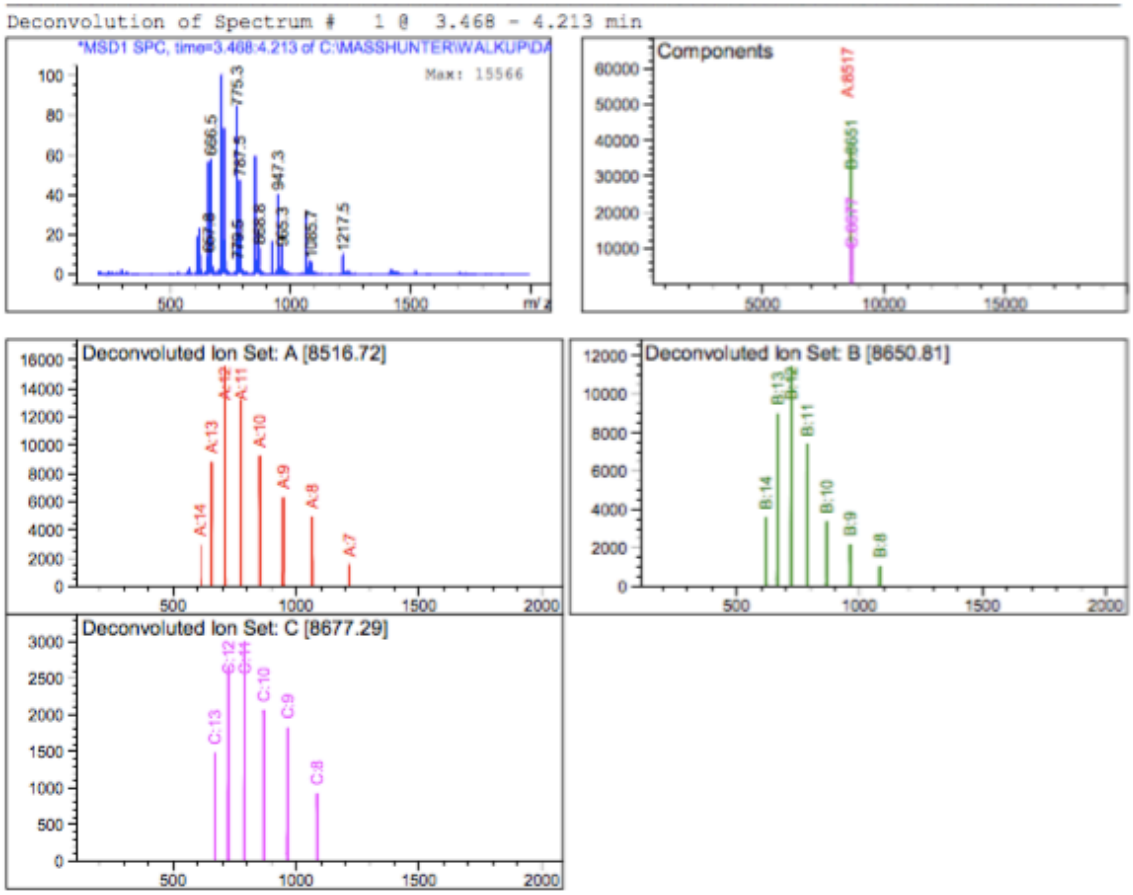
Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8516.59	72789	100.00
B	8651.12	47457	65.20

Figure S58: Protein LCMS trace and deconvolution of purified **17g** showing only two species present, the total ion of product **17g** (8651 Da) and the fragment ion for **17g**-134 (8517 Da). The fact that **17g** was separated on an IEX column eludes to the fact that the fragment ion must be an artefact of the mass spectrometer, as a real species of this nature would elute at a dramatically different retention time on an ion exchange column.



Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8564.78	477342	100.00

Figure S59: Protein LCMS trace and deconvolution of purified parent Ubiquitin

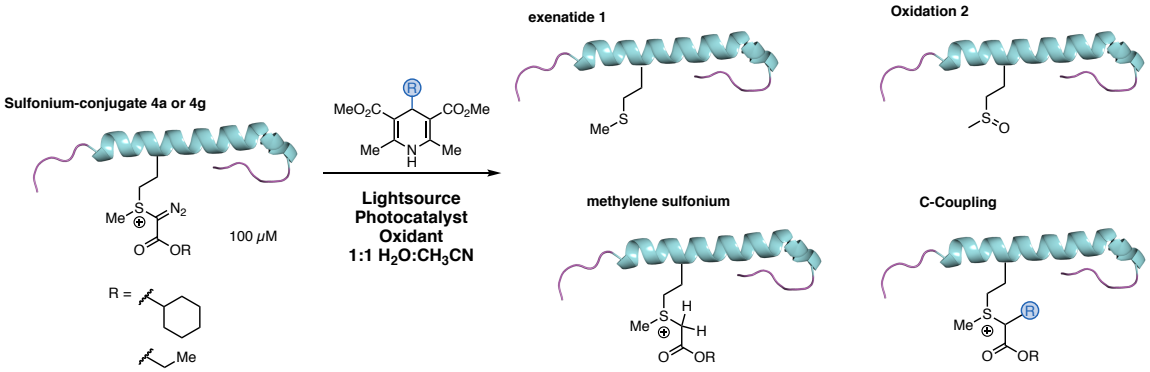


Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8516.72	61187	100.00
B	8650.81	37658	61.55
C	8677.29	11755	19.21

Figure S60: Protein LCMS trace of mixed fraction of **11** and **17g** showing the total ion of product **17g** (8651 Da) and the total ion for **11** (8677). Additionally, the same fragment ion for **17g**-134 and **11**-160 (8517 Da) is observed. Again, were this not an artefact of ionization in the mass spectrometer, this uncharged species would elute at a dramatically different retention time on the IEX column.

Photochemical Alkylation

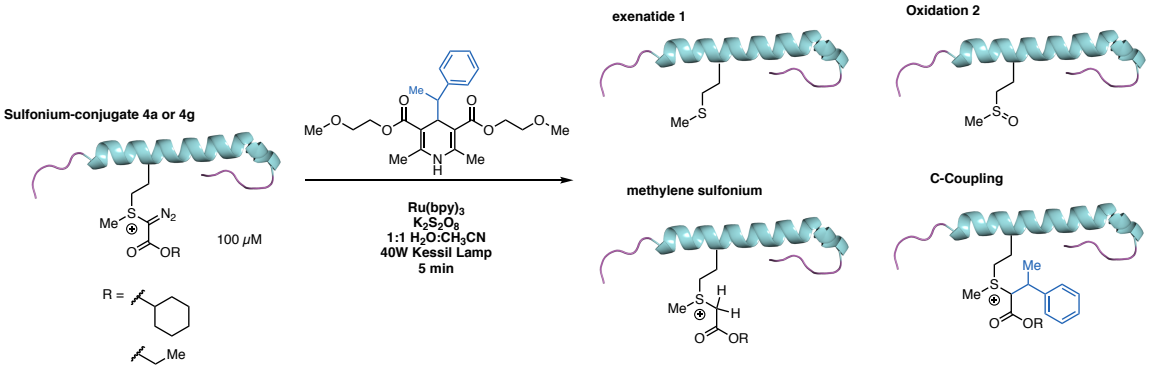
Table S4. Preliminary Optimization of the Photochemical alkylation Process.



Entry	R	[Hantzsch]	Photocatalyst	Oxidant	Light[a]	T(min)	%conversion	% Free peptide	%oxidized peptide	% CH_2	% C-alk
1[b,c]		20 mM	$Ir(dtbpy)(ppy)_2$ (200 μM)	—	30W	30	>95%	13	0	77	10
2[b,c]		20 mM	$Ru(bpy)_3$ (200 μM)	$K_2S_2O_8$ (2.5 mM)	30W	30	87	21	5	16	15
3[b,d]		20 mM	$Ir(dtbpy)(ppy)_2$ (50 μM)	—	40W Kessil	15	95%	12	0	75	13
4[b,d]		20 mM	$Ru(bpy)_3$ (200 μM)	$K_2S_2O_8$ (2.5 mM)	30W	30	87	16	7	35	13
5[b,d]		20 mM	$Ru(bpy)_3$ (200 μM)	—	40W Kessil	15	>95%	22	7	41	31
6[b,d]		40 mM	$Ru(bpy)_3$ (300 μM)	$K_2S_2O_8$ (2.5 mM)	40W Kessil	15	>95%	17	trace	50	32
7[b,d]		40 mM	$Ru(bpy)_3$ (200 μM)	$K_2S_2O_8$ (10mM)	40W Kessil	5	94	11	7	28	38
8[b,d]		30 mM	$Ru(bpy)_3$ (200 μM)	$K_2S_2O_8$ (10mM)	40W Kessil	5	94	0	17	83	0

[a]Light placed at 5 cm distance from reaction vessel. [b]Reaction outcome determined by TIC. [c]ethyl ester. [d]cyclohexyl ester

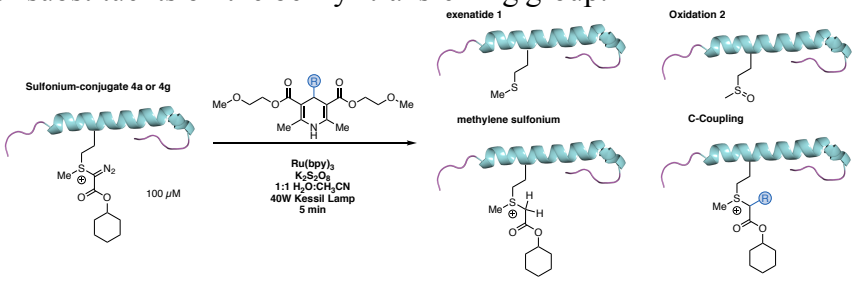
Table S5. Optimization of the Photochemical alkylation Process using **S-12** as a Hantzsch ester with enhanced solubility characteristics.



Entry	[Hantzsch]	[Ru(bpy)$_3$]	[$K_2S_2O_8$]	%conversion	% Free peptide	%oxidized peptide	% CH_2	% C-alk
1[b,c]	40 mM	200 μM	10 mM	>95	12	6	41	25
2[b,c]	40 mM	—	10 mM	36	2	4	27	trace
3[b,d]	40 mM	500 μM	10 mM	>95	15	trace	29	44
4[b,d]	40 mM	25 μM	10 mM	95	12	5	33	29
5[b,d]	40 mM	500 μM	10 mM	>95	18	3	22	42
6[b,d]	40 mM	1 mM	10 mM	>95	19	trace	19	48
7[b,d]	40 mM	2 mM	10 mM	>95	16	trace	27	42
7[b,d]	40 mM	1 mM	20 mM	>95	18	18	13	47
7[b,d]	20 mM	1 mM	10 mM	>95	23	trace	10	53

[a]Light placed at 5 cm distance from reaction vessel. [b]Reaction outcome determined by TIC. [c]ethyl ester. [d]cyclohexyl ester

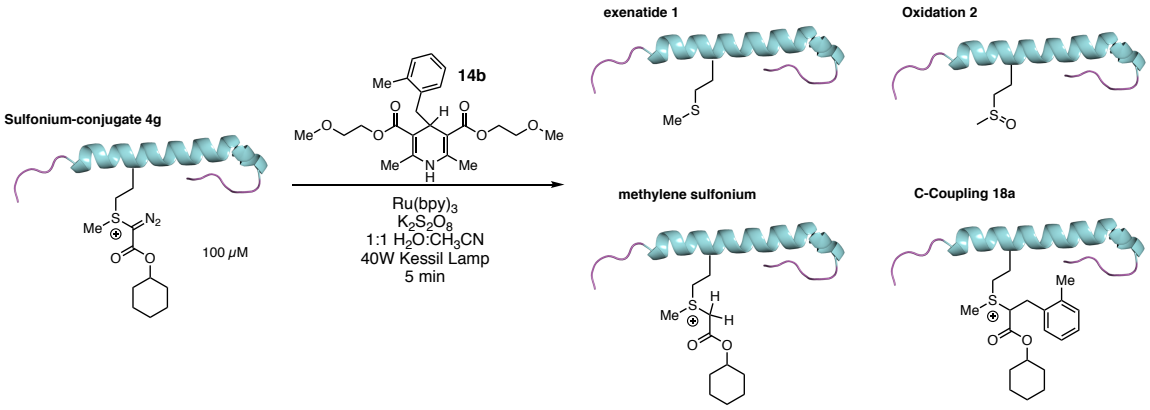
Table S6. Optimization of the Photochemical alkylation Process by systematic variance of substituents on the benzyl-transferring group.



Entry	R	[Hantzsch]	[$Ru(bpy)_3$]	[$K_2S_2O_8$]	%conversion	% Free peptide	%oxidized peptide	% CH_2	% C-alk	Alk/CH_2 ratio
1[b,c]		20 mM	1 mM	10 mM	>95	trace	0	28	72	**2.6/1**
2[b,c]		10 mM	500 μM	10 mM	36	12	8	36	45	**1.3/1**
4[b,d]		40 mM	1 mM	10 mM	>95%	12	0	88*	0	-
5[b,d]		20 mM	1 mM	10 mM	>95%	13	6	68	13	**0.2/1**
6[b,d]		30 mM	1 mM	10 mM	>95%	18	0	62*	20	**0.3/1**
7[b,d]		40 mM	500 μM	10 mM	92%	5	0	79	8	**0.1/1**
8[b,d]		20 mM	1 mM	10 mM	>95%	Decomposition				
9[b,d]		40 mM	500 μM	10 mM	>95%	Decomposition				
10[b,d]		20 mM	1 mM	10 mM	>95%	Decomposition				
11[b,d]		10 mM	500 μM	5 mM	67%	6	trace	16	45	**2.9/1**
13[b,d]		20 mM	1 mM	10 mM	>95%	5	4	36	54	**1.5/1**
14[b,d]		10 mM	500 μM	5 mM	>95%	trace	4	43	51	**1.2/1**
15[b,d]		20 mM	1 mM	10 mM	>95%	8	trace	19	72	**3.2/1**
16[b,d,e]		30 mM	1 mM	10 mM	>95%	3	trace	7	86	**12/1**
17[b,d]		20 mM	1 mM	10 mM	>95%	0	0	22	70	**3.2/1**
18[b,d]		30 mM	1 mM	10 mM	89%	6	0	9	72	**8.0/1**
19[b,d]		30 mM	1 mM	10 mM	53%	8	3	11	32	**3.0/1**
20[b,d]		20 mM	1 mM	10 mM	71%	10	4	16	37	**0.8/1**

[a]Light placed at 5 cm distance from reaction vessel. [b]Reaction outcome determined by TIC. [c]ethyl ester. [d]cyclohexyl ester [e] Average of 5 runs * Reduction includes some reduction + ox.

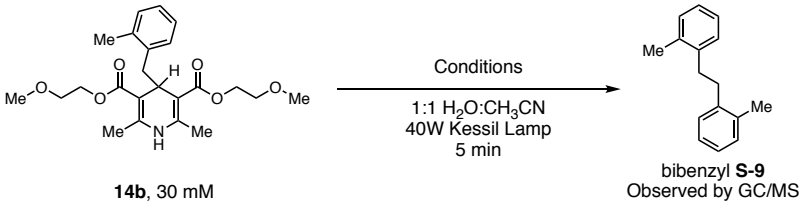
Table S7. Control reactions to assist in identifying the role of each reagent.



Entry	[Hantzsch]	[Ru(bpy)$_3$]	[$K_2S_2O_8$]	Light[a]	%conversion[b]	% Free peptide	%oxidized peptide	% CH_2	% C-alk
1[c]	30 mM	1 mM	10 mM	40W kessil	>95	3	trace	7	86
2	30 mM	1 mM	—	40W kessil	56	3	trace	44	4
3	30 mM	—	10 mM	40W Kessil	25	3	7	14	trace
4	30 mM	1 mM	10 mM	dark[d]	Decomposition				

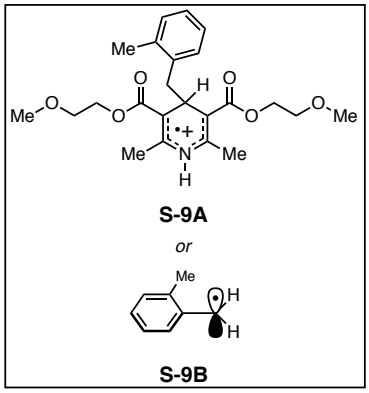
[a]Light placed at 5 cm distance from reaction vessel. [b]Reaction outcome determined by TIC. [c] Values are an average of 5 experiments. [d]reaction was run in a foil-encapsulated vial.

Table S8. Control reactions to assist in identifying the role of each reagent. Conditions that form bibenzyl **S-9** would suggest the formation of either of two radical species **S-9A** or **S-9B**.



Entry	[Ru(bpy)$_3$]	[$K_2S_2O_8$]	Light[a]	S-9 Observed?
1	1 mM	10 mM	40W kessil	Yes
2	1 mM	—	40W kessil	No
3	—	—	40W Kessil	No

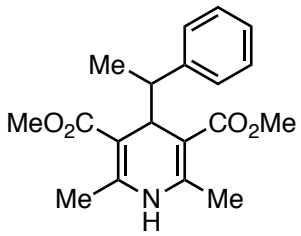
[a]Light placed at 5 cm distance from reaction vessel



General Procedure D: Synthesis of C-4 Substituted Hantzsch esters

A solution of aldehyde (1 eq.), 2-methoxyethyl 3-oxobutanoate (4 eq.) and ammonium acetate (2 eq.) in water (0.5 M in aldehyde) was stirred at reflux under air for 4 hours and cooled to room temperature. The reaction mixture was diluted with EtOAc and the aqueous layer extracted with EtOAc (x 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and evaporated to yield the crude product. The crude product was purified by silica column chromatography to yield the desired Hantzsch ester.

Dimethyl 2,6-dimethyl-4-(1-phenylethyl)-1,4-dihydropyridine-3,5-dicarboxylate (S-10)

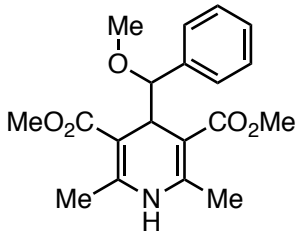


S-10 was prepared according to General Procedure D using (2-phenyl propanal (2.7 mL, 20 mmol), methyl acetoacetate 9.2 mL, 80 mmol), and ammonium acetate (3.11 g, 40 mmol), with a reaction time of 5 hours. After workup, the crude residue was recrystallized twice from ethyl acetate/40-60 petroleum ether at –20° C to yield S-10 (3.17 g, 48%) as colorless crystals.

MP: 147–150 °C
^{1}H-NMR (500 MHz, 6:1 CD_3CN:$CDCl_3$) δ: 7.22-7.16 (m, 2H), 7.15-7.10 (m, 1H), 7.09-7.04 (m, 2H), 6.80 (s, br, 1H), 4.13 (d, *J* = 6.1 Hz, 1H), 3.47 (s, 3H), 3.44 (s, 3H), 2.62 (app quint, *J* =7.1, 1H), 2.18 (s, 3H), 2.16 (s, 3H), 1.09 (d, *J* = 7.2 Hz, 3H).
^{13}C-NMR (125 MHz, 6:1 CD_3CN:$CDCl_3$) δ: 168.5, 168.3, 146.2 (2C), 144.4, 128.3 (2C), 127.2 (2C), 125.7, 100, 99.6, 50.1, 50.0, 45.8, 40.1, 17.6 (2C), 15.0.
IR: 3325, 3092, 3026, 2967, 2939, 1697, 1658, 1636, 1487, 1431, 1341, 1214, 1189, 1102, 1005, 876, 755.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 330.1698, Calc'd $C_{19}H_{24}NO_4$ requires 330.1700.

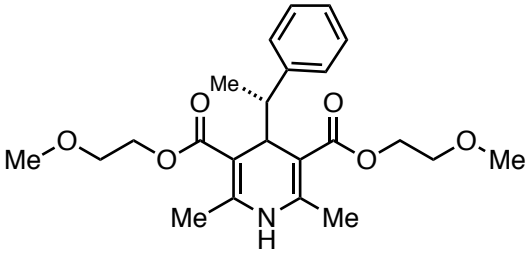
Dimethyl 4-(methoxy(phenyl)methyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-11)

S-11 was prepared according to General Procedure D using (2-methoxy-2-phenyl acetaldehyde[14] (0.278 g, 1.86 mmol), methyl acetoacetate 0.80 mL, 7.4 mmol), and ammonium acetate (0.289 g, 3.72 mmol), with a reaction time of 8 hours. After workup, the crude residue was recrystallized once from ethyl acetate/40-60 petroleum ether at –20° C to yield **S-11** (0.331 g, 52%) as off-white crystals.



MP: 144-145 °C
^{1}H-NMR (500 MHz, CD_3CN) δ: 7.27-7.19 (m, 3H), 7.11-7.06 (m, 2H), 6.66 (s, br, 1H), 4.35 (d, *J* = 6.2 Hz, 1H), 3.86 (d, *J* = 6.0 Hz, 1H), 3.57 (s, 3H), 3.42 (s, 3H), 3.10 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 168.4, 168.3, 146.3, 146.1, 139.6, 127.7, 127.0, 98.5, 98.0, 86.8, 56.3, 50.2, 49.9, 40.0, 17.4, 17.2.
IR: 3337 (br). 3086, 2950, 2816, 1696, 1654, 1486, 1433, 1297, 1208, 1185, 1093, 1022, 761.
HRMS-$(ESI)^+$ (m/z) Found (M+Na): 368.1475, Calc'd $C_{19}H_{23}NO_5Na$ requires 368.1468.

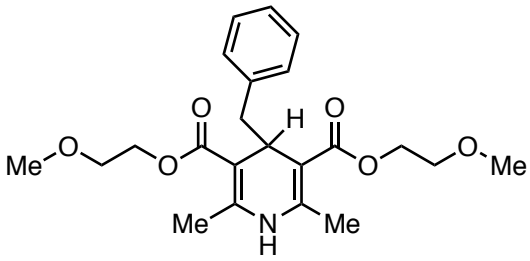
bis(2-methoxyethyl) (*S*)-2,6-dimethyl-4-(1-phenylethyl)-1,4-dihydropyridine-3,5-dicarboxylate (S-12)



S-12 was prepared according to General Procedure D using (2-phenyl propanal (1.4 mL, 10 mmol), 2-methoxyethyl acetoacetate (5.9 mL, 40 mmol), and ammonium acetate (1.542 g, 20.0 mmol), with a reaction time of 8 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-12** (2.287 g, 55%) as a golden syrup.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.24-7.18 (m, 2H), 7.17-7.11 (m, 3H), 5.39 (s, br, 1H), 4.33 (app d, *J* = 4.8 Hz, 1H), 4.20-4.08 (m, 3H), 3.95-3.88 (m, 1H), 3.63 (app t, *J* = 4.9 Hz, 2H), 3.53-3.47 (m, 2H), 3.43 (s, 3H), 3.40 (s, 3H), 2.86-2.77 (m, 1H), 2.22 (s, 3H), 2.21 (s, 3H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 168.0, 167.8, 146.4, 146.2, 144.5, 128.4 (2C), 127.2 (2C), 125.7, 99.9, 99.6, 70.4, 70.3, 62.3 (2C), 58.0 (2C), 45.9, 40.1, 17.6, 17.5, 14.9.
IR: 3331, 2936, 2878, 1691 (br), 1618, 1488, 1450, 1373, 1302, 1271, 1206, 1087, 1023, 864, 769.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 418.2231, Calc'd $C_{23}H_{32}O_6N_2$ requires 418.2224

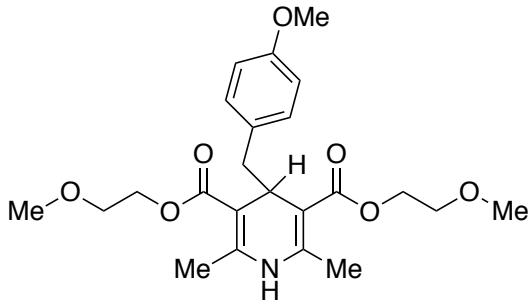
bis(2-methoxyethyl) 4-benzyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-13)



S-13 was prepared according to General Procedure D using phenylacetaldehyde (1.17 mL, 10 mmol), 2-methoxyethyl acetoacetate (6.0 mL, 40 mmol), and ammonium acetate (1.55 g, 20 mmol), with a reaction time of 3 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-13** (2.02 g, 50%) as a brown oil of >85% purity. S-13 can be slowly crystallized from diethyl ether/40-60 petroleum ether at –20°C.

MP: 49–52 °C
^{1}H-NMR (500 MHz, CD_3CN) δ: 7.20-7.12 (m, 3H), 7.04-6.99 (m, 2H), 6.67 (s, br, 1H), 4.13-4.06 (m, 3H), 4.03-3.98 (m, 2H), 3.55-3.47 (m, 4H), 3.34 (s, 6H), 2.52 (d, J = 2.5 Hz, 2H), 2.13 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.4 (2C), 146.7 (2C), 139.4, 130.0 (2C), 127.3 (2C), 125.5, 100.5 (2C), 70.4 (2C), 62.3 (2C), 58.0 (2C), 42.0, 35.5, 17.6 (2C).
IR: 3338 (br), 3029, 2924, 2889, 2843, 2818, 1675 (br), 1641, 1617, 1488, 1451, 1381, 1300, 1266, 1202, 1176, 1091, 1058, 1026, 982, 854, 751.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 404.2030, Calc'd $C_{22}H_{30}NO_6$ requires 404.2068

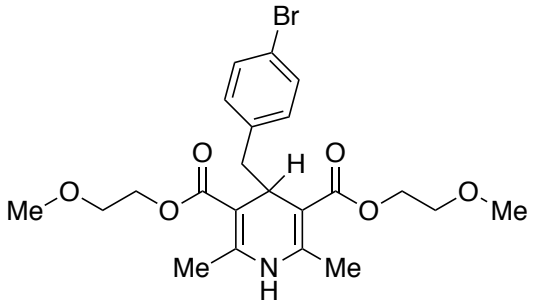
Bis(2-methoxyethyl) 4-(4-methoxybenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-14)



S-14 was prepared according to General Procedure D using 2-(4-methoxyphenyl) acetaldehyde (0.98 g, 6.5 mmol), 2-methoxyethyl 3-oxobutanoate (3.8 g, 26.0 mmol) and ammonium acetate (1.0 g, 13.0 mmol) in water (15 mL), with a reaction time of 4 hours. Purification by flash silica column chromatography (20-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/hexane afforded **S-14** (0.97 g, 34%) as an off-white solid.
MP: 62 - 64 °C (decomp)
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.94 (d, J = 8.3 Hz, 2H), 6.72 (d, J = 8.3 Hz, 2H), 5.18 (br. s, 1H), 4.23 – 4.12 (m, 5H), 3.76 (s, 3H), 3.61 – 3.54 (m, 4H), 3.40 (s, 6H), 2.55 (d, J = 5.4 Hz, 2H), 2.16 (s, 6H).
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.8 (2C), 158.0, 145.8, 131.56 (2C), 131.2 (2C), 112.9 (2C), 101.8 (2C), 70.8 (2C), 62.8 (2C), 59.1 (2C), 55.4, 41.4, 35.7, 19.4 (2C).

IR: 3337, 2985, 2941, 2862, 2885, 2838, 1684, 1653, 1625, 1609, 1497, 1470, 1445, 1224, 1212, 1089, 1029, 856, 796, 697.
HRMS-(ESI)$^+$ (m/z) Found (M+H): 434.2176, Calc'd $C_{23}H_{32}O_7N$ requires 434.2173.
Rf: 0.3 (50% EtOAc:40-60 Petroleum ether)

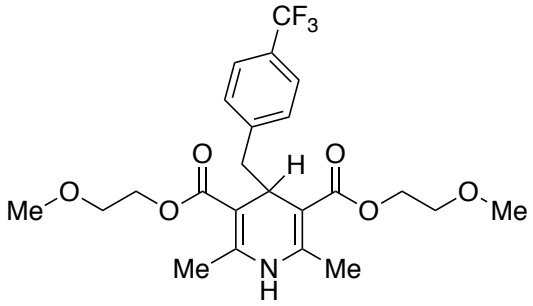
Bis(2-methoxyethyl) 4-(4-bromobenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-15)



S-15 was prepared according to General Procedure D using 2-(4-bromophenyl) acetaldehyde (0.80 g, 4.0 mmol), 2-methoxyethyl 3-oxobutanoate (2.34 g, 16.0 mmol) and ammonium acetate (0.62 g, 2.0 mmol) in water (8 mL), with a reaction time of 4 hours. Purification by flash silica column chromatography (10-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/hexane afforded **S-15** (0.47 g, 24%) as an off-white solid.

MP: 112 - 114 °C (decomp)
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.29 (d, *J* = 8.1 Hz, 2H), 6.91 (d, *J* = 8.1 Hz, 2H), 5.23 (br. s, 1H), 4.23 – 4.15 (m, 5H), 3.62 – 3.51 (m, 4H), 3.40 (s, 6H), 2.57 (d, *J* = 5.4 Hz, 2H), 2.17 (s, 6H)
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.6 (2C), 146.0 (2C), 138.6, 132.0 (2C), 130.4 (2C), 119.8, 101.6 (2C), 70.8 (2C), 62.8 (2C), 59.1 (2C), 41.7, 35.6, 19.5 (2C).
IR: 3338, 3043, 2981, 2879, 2811, 1671, 1654, 1481, 1373, 1224, 1123, 1024, 1009, 770.
HRMS-(ESI)$^+$ (m/z) Found (M+H): 484.1162, Calc'd $C_{22}H_{29}{}^{81}BrO_6N$ requires 484.1152.
Rf: 0.4 (50% EtOAc:40-60 Petroleum ether)

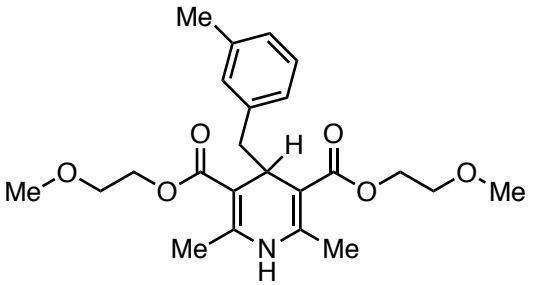
Bis(2-methoxyethyl) 2,6-dimethyl-4-(4-(trifluoromethyl)benzyl)-1,4-dihydropyridine-3,5-dicarboxylate (S-16)



S-16 was prepared according to General Procedure D using 2-(4-(trifluoromethyl)phenyl) acetaldehyde (0.75 g, 4.0 mmol), 2-methoxyethyl 3-oxobutanoate (2.34 g, 16.0 mmol) and ammonium acetate (0.62 g, 2.0 mmol) in water (8 mL), with a reaction time of 4 hours. Purification by flash silica column chromatography (10-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/hexane afforded **S-16** (0.60 g, 32%) as an off-white solid.

MP: 102 - 104 °C (decomp)
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.43 (d, *J* = 7.9 Hz, 2H), 7.16 (d, *J* = 7.9 Hz, 2H), 5.33 (br. s, 1H), 4.25 (t, *J* = 5.6 Hz, 1H), 4.21 – 4.07 (m, 4H), 3.60 – 3.50 (m, 4H), 3.39 (s, 6H), 2.67 (d, *J* = 5.6 Hz, 2H), 2.18 (s, 6H).
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.5 (2C), 146.0 (2C), 143.9, 130.5 (2C), 128.1 (q, *J* = 32.6 Hz), 124.6 (q, *J* = 270.2 Hz), 124.2 (2C), 101.6 (2C), 70.8 (2C), 62.9 (2C), 59.1 (2C), 42.4, 35.7, 19.4 (2C).
^{19}F-NMR (376 MHz, $CDCl_3$) δ: -64.3.
IR: 3339, 2934 (br), 2885 (br), 1673, 1652, 1615, 1477, 1323, 1224, 1165, 1099, 1089, 1026, 1015, 859, 773, 709.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 472.1949, Calc'd $C_{23}H_{29}F_3O_6N$ requires 472.1941
Rf: 0.4 (50% EtOAc:40-60 Petroleum ether)

bis(2-methoxyethyl) 2,6-dimethyl-4-(3-methylbenzyl)-1,4-dihydropyridine-3,5-dicarboxylate (S-17)

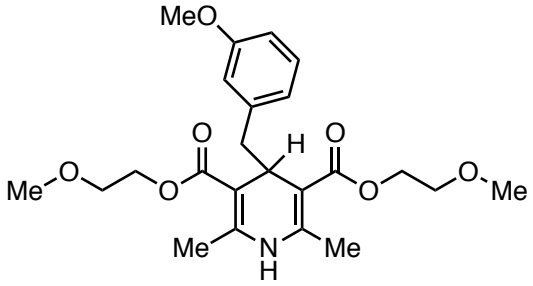


S-17 was prepared according to General Procedure D using *m*-tolyl acetaldehyde (0.40 g, 3.1 mmol), 2-methoxyethyl acetoacetate (1.8 mL, 12 mmol), and ammonium acetate (0.47 g, 6.0 mmol), with a reaction time of 3 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-17** (0.365 g, 29%) as a faint yellow, sticky oil of >80% purity that solidified upon standing. S-17 can be further crystallized from diethyl ether/40-60 petroleum ether at –20°C.

MP: 51-52 °C

^{1}H-NMR (500 MHz, CD_3CN) δ: 7.07-7.03 (m, 1H), 6.99-6.94 (m, 1H), 6.85 (s, 1H), 6.81-6.77 (m, 1H), 6.69 (s, br, 1H), 4.12-4.06 (m, 3H), 4.02-3.97 (m, 2H), 3.54-3.47 (m, 4H), 3.34 (s, 6H), 2.47 (d, *J* = 5.75 Hz, 2H*),* 2.27 (s, 3H), 2.14 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.4 (2C), 146.7 (2C), 139.3, 136.7, 130.8, 127.2, 127.1, 126.1, 100.6, 70.4 (2C), 62.3 (2C), 58.0 (2C), 42.0, 35.5, 20.5, 17.6 (2C).
IR: 3344 (br), 2890, 2815, 1688, 1674, 1645, 1620, 1487, 1455, 1383, 1301, 1268, 1207, 1090, 1026, 854, 770.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 418.2229, Calc'd $C_{23}H_{32}NO_6$ requires 418.2224.

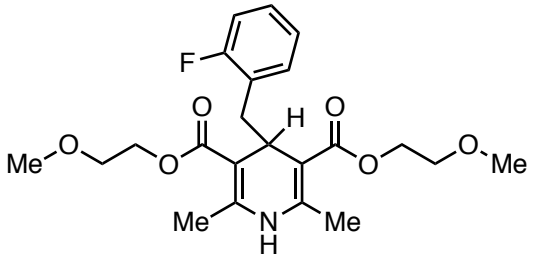
bis(2-methoxyethyl) 4-(3-methoxybenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-18)



S-18 was prepared according to General Procedure D using *m*-methoxyphenyl acetaldehyde (0.41 g, 2.8 mmol), 2-methoxyethyl acetoacetate (1.6 mL, 11 mmol), and ammonium acetate (0.43 g, 5.5 mmol), with a reaction time of 3 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-18** (0.61 g, 51%) as a yellow oil.

^{1}H-NMR (500 MHz, CD_3CN) δ: 7.09 (app t, *J* = 7.9 Hz, 1H), 6.73 (s, br, 1H), 6.70 (app ddd, *J* = 1.0, 2.7, 8.2 Hz, 1H), 6.63-6.60 (m, 1H), 6.59-6.57 (m, 1H), 4.12-4.07 (m, 3H), 4.03-3.98 (m, 2H), 3.74 (s, 3H), 3.54-3.47 (m, 4H), 3.33 (s, 6H), 2.49 (d, *J* = 5.9 Hz, 1H), 2.15 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.4 (2C), 159.0, 146.7 (2C), 141.0, 128.2, 122.4, 115.3, 111.3, 100.7 (2C), 70.4 (2C), 62.3 (2C), 58.0 (2C), 54.7, 42.1, 35.5, 17.6 (2C).
IR: 3331 (br), 2931 (br), 1691 (br), 1646, 1600, 1487, 1452, 1380, 1269, 1210, 1103, 1052, 861, 772,
HRMS-$(ESI)^+$ (m/z) Found (M+H): 434.2144, Calc'd $C_{23}H_{32}NO_7$ requires 434.2173.

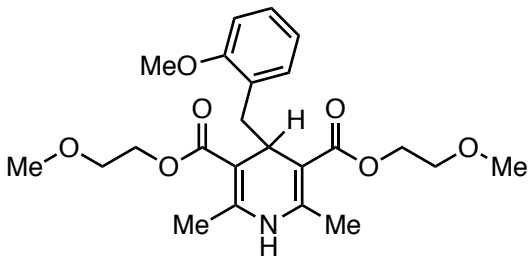
bis(2-methoxyethyl) 4-(2-fluorobenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-19)



S-19 was prepared according to General Procedure D using *o*-fluorophenyl acetaldehyde (0.42 g, 3.1 mmol), 2-methoxyethyl acetoacetate (1.8 mL, 12 mmol), and ammonium acetate (0.46 g , 6.0 mmol), with a reaction time of 3 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-19** (0.48 g, 37%) as a faint yellow, sticky oil of >90% purity that solidified upon standing. S-19 can be further crystallized from diethyl ether/40-60 petroleum ether at –20°C.

MP: 62–64 °C
^{1}H-NMR (500 MHz, CD_3CN) δ: 7.21-7.14 (m, 1H), 7.06-6.92 (m, 3H), 6.70 (s, br, 1H), 4.19 (t, *J* = 5.7 Hz, 1H), 4.12 (app t, *J* = 5.0 Hz, 1H), 4.10 (app dd, *J* = 4.4, 5.6 Hz, 1H), 3.03 (app dd, *J* =4.3, 5.2 Hz, 1H), 4.01 (app t, *J* = 4.7 Hz, 1H), 3.56-3.51 (m, 4H), 3.33 (s, 6H), 2.60 (app dd, *J* = 1.0, 5.6 Hz, 2H), 2.14 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.2 (2C), 161.8 (d, *J*= 243 Hz), 146.9 (2C), 132.8 (d, *J*= 5.3 Hz), 127.7 (d, *J*= 8.6 Hz), 126.1 (d, *J*=16.8 Hz), 123.2 (d, *J*= 3 Hz), 114.2 (d, *J*= 22.6 Hz), 100.2 (2C), 70.4 (2C), 62.4 (2C), 58.0 (2C), 34.5 (2C), 17.6 (2C).
^{19}F-NMR (376 MHz, CD_3CN) δ -119.3 (s).
IR: 3328, 2954, 2884, 2840, 2821, 1688, 1644, 1619, 1490, 1438, 1379, 1273, 1212, 1078, 1057, 1019, 859, 757.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 422.1978, Calc'd $C_{22}H_{29}FNO_6$ requires 422.1973.

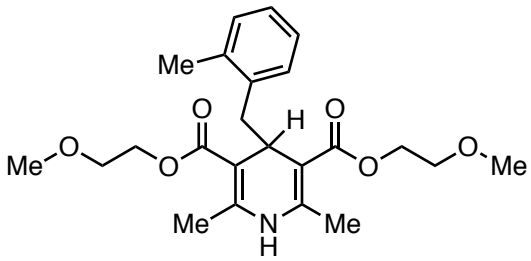
bis(2-methoxyethyl) 4-(2-methoxybenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-20)



S-20 was prepared according to General Procedure D using (*o*-methoxyphenyl) acetaldehyde (0.45 g, 3.0 mmol), 2-methoxyethyl acetoacetate (1.8 mL, 12 mmol), and ammonium acetate (0.47 g, 6.0 mmol), with a reaction time of 3 hours. Column chromatography (20-50% ethyl acetate/40-60 petroleum ether) afforded **S-20** (0.651 g, 50%) as a faint yellow solid of >90% purity that solidified upon standing. S-20 can be further crystallized from diethyl ether/40-60 petroleum ether at –20°C.

MP: 87–79 °C
^{1}H-NMR (500 MHz, CD_3CN) δ: 7.15-7.10 (m, 1H), 6.90 (app dd, *J* = 1.7, 7.4 Hz, 1H), 6.82-6.78 (m, 1H), 6.75 (app dt, *J* = 1.3, 7.4 Hz, 1H), 6.57 (s, br, 1H), 4.16 (app t, *J* = 5.6 Hz, 1H), 4.15-4.12 (m, 1H), 4.11 (app dd, *J* = 4.3, 5.5 Hz, 1H), 4.04 (app dd, *J* = 4.2, 5.3 Hz, 1H), 4.01 (app t, *J* = 4.7 Hz, 1H), 3.71 (s, 3H), 3.56-3.51 (m, 4H), 3.34 (s, 6H), 2.57 (d, *J* = 5.6 Hz, 2H), 2.11 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.5 (2C), 158.2, 146.4 (2C), 131.7, 127.5, 127.0, 119.3, 109.6, 100.7, 70.5 (2C), 62.3 (2C), 58.0 (2C), 54.9, 35.1, 34.6, 17.6 (2C).
IR: 3328 (br), 2991, 2940, 2876, 2842, 2810, 1693, 1652 (br), 1481, 1379, 1323, 1300, 1269, 1243, 1211, 1091, 1057, 867, 750.
HRMS-$(ESI)^+$ (m/z) Found (M+Na): 456.1788, Calc'd $C_{23}H_{31}NO_7Na$ requires 456.1993.

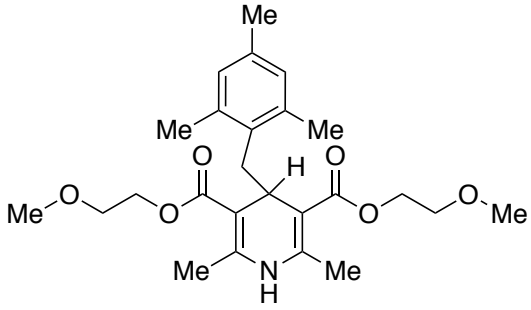
bis(2-methoxyethyl)-2,6-dimethyl-4-(2-methylbenzyl)-1,4-dihydropyridine-3,5-dicarboxylate (14b)



14b was prepared according to General Procedure D using *o*-tolyl acetaldehyde (0.81 g, 6.01 mmol), 2-methoxyethyl acetoacetate (3.5 mL, 24.1 mmol), and ammonium acetate (0.94 g, 12.0 mmol), with a reaction time of 3 hours. After workup, the crude residue was recrystallized from 1:1 diethyl ether/40-60 petroleum ether at –20° C to yield 14b (1.07 g, 43%) as colorless crystals.

MP: 79-80°C
^{1}H-NMR (500 MHz, CD_3CN) δ: 7.09-7.00 (m, 3H), 6.97 (app td, *J* = 1.4, 7.3 Hz, 1H), 6.67-6.61 (app dd, *J* = 1.4, 7.4 Hz, 1H), 4.16 (t, *J* = 6.9 Hz, 1H), 4.03 (app dd, *J* = 4.8, 5.8 Hz, 1H), 4.07 (app dd, *J* = 4.4, 5.9 Hz, 1H), 3.81 (app dd, *J* =4.1, 5.1 Hz, 1H), 3.79 (app t, *J* = 5 Hz, 1H), 3.5-3.42 (m, 4H), 3.31 (s, 6H), 2.52 (d, *J* = 7Hz, 2H), 2.35 (s, 3H), 2.24 (s, 6H).
^{13}C-NMR (125 MHz, CD_3CN) δ: 167.3 (2C), 146.6 (2C), 137.2 (2C), 131.1, 129.5, 125.7, 124.8, 101.2(2C), 70.3 (2C), 62.3(2C), 58.0, 39.1, 33.5, 18.6, 17.7 (2C)
IR: 3331 (br), 3076, 2896 (br), 2820, 1692, 1641, 1619, 1486, 1455, 1379, 1309, 1265, 1202, 1104, 1086, 1020, 744.
HRMS-$(ESI)^+$ (m/z) Found (M+H): 418.2232, Calc'd $C_{23}H_{32}O_6N_2$ requires 418.2224.

Bis(2-methoxyethyl) 2,6-dimethyl-4-(2,4,6-trimethylbenzyl)-1,4-dihydropyridine-3,5-dicarboxylate, S-21



S-21 was prepared according to General Procedure D using 2-mesitylacetaldehyde (0.32 g, 2 mmol), 2-methoxyethyl 3-oxobutanoate (1.17 g, 8 mmol) and ammonium acetate (0.31 g, 4 mmol) in water (4 mL), with a reaction time of 6 hours. Purification by flash silica column chromatography (10-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/EtOAc/hexane afforded **S-21** (0.16 g, 18%) as an off-white solid.

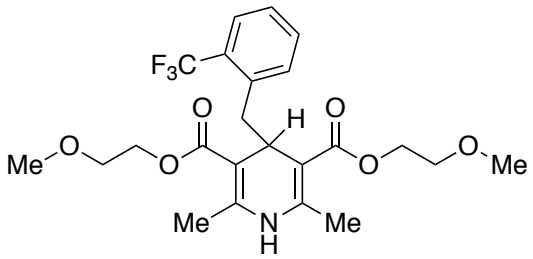
MP: 110 °C (decomp)
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.75 (s, 2H), 5.71 (br. s, 1H), 4.34 (t, *J* = 7.9 Hz, 1H), 4.06 (dt, *J* = 4.8, 12.0 Hz, 2H), 3.82 (dt, *J* = 4.8, 12.0 Hz, 2H), 3.48 (t, *J* = 4.8 Hz, 4H), 3.36 (s, 6H), 2.66 (d, *J* = 7.9 Hz, 2H), 2.31 – 2.28 (m, 12H), 2.19 (s, 3H).
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.8 (2C), 145.3 (2C), 137.8 (1C or 2C), 135.0 (1C or 2C), 132.2 (1C or 2C), 128.7 (2C), 103.4 (2C), 70.6 (2C), 62.7 (2C), 59.0 (2C), 35.2 (1C), 32.7 (1C), 20.9 (1C), 20.1 (2C), 19.6 (2C).

IR: 3303, 2912 (br), 2806, 1701, 1690, 1619, 1491, 1305, 1266, 1198, 1084, 1058, 1017, 847, 773, 766, 721.

HRMS-$(ESI)^+$ (m/z) Found (M+H): 446.2515, Calc'd $C_{25}H_{36}O_6N$ requires 446.2537.

Rf: 0.4 (50% EtOAc:40-60 Petroleum ether)

Bis(2-methoxyethyl) 2,6-dimethyl-4-(2-(trifluoromethyl)benzyl)-1,4-dihydropyridine-3,5-dicarboxylate (S-22)



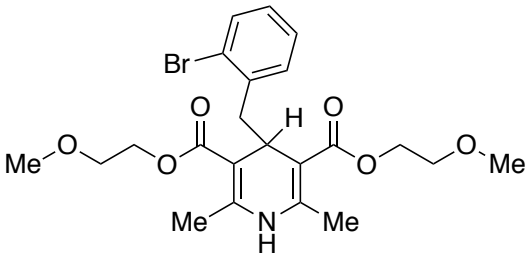
S-22 was prepared according to General Procedure D using 2-(2-(trifluoromethyl)phenyl)acetaldehyde (0.38 g, 2 mmol), 2-methoxyethyl 3-oxobutanoate (1.17 g, 8 mmol) and ammonium acetate (0.31 g, 4 mmol) in water (4 mL), with a reaction time of 6 hours. Purification by flash silica column chromatography (10-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/hexane afforded **S-22** (0.35 g, 37%) as an off-white solid.

MP: 76 °C (decomp)
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.56 (d, *J* = 7.6 Hz, 1H), 7.38 (t, *J* = 7.6 Hz, 1H), 7.23 (t, *J* = 7.6 Hz, 2H), 5.85 (br. s, 1H), 4.36 (t, *J* = 7.3 Hz, 1H), 4.13 = 4.06 (m, 2H), 3.91 – 3.84 (m, 2H), 3.53 – 3.44 (m, 4H), 3.36 (s, 6H), 2.76 (d, *J* = 7.3 Hz, 2H), 2.32 (s, 6H).
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.5 (2C), 146.1 (2C), 137.9, 133.5, 130.8, 129.14 (q, *J* = 30.0 Hz), 125.9, 125.6 (q, *J* = 5.7 Hz), 124.8 (q, *J* = 275.1 Hz), 102.5 (2C), 70.5 (2C), 62.7 (2C), 59.0 (2C), 38.4, 34.9, 19.6 (2C).
^{19}F-NMR (376 MHz, $CDCl_3$) δ: -60.9.

IR: 3327, 2928 (br), 2821, 1691, 1639, 1617, 1489, 1310, 1265, 1206, 1206, 1157, 1112, 1089, 1057, 1032, 771, 744.

HRMS-$(ESI)^+$ (m/z) Found (M+H): 472.1925, Calc'd $C_{23}H_{29}F_3O_6N$ requires 472.1941.
Rf: 0.3 (50% EtOAc:40-60 Petroleum ether)

Bis(2-methoxyethyl) 4-(2-bromobenzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (S-23)



S-23 was prepared according to General Procedure D using 2-(2-bromophenyl)acetaldehyde (0.40 g, 2 mmol), 2-methoxyethyl 3-oxobutanoate (1.17 g, 8 mmol) and ammonium acetate (0.31 g, 4 mmol) in water (4 mL), with a reaction time of 5 hours. Purification by flash silica column chromatography (10-50% EtOAc/40-60 petroleum ether) followed by recrystallization from diethyl ether/hexane afforded **S-23** (0.36 g, 37%) as an off-white solid.

MP: 68 - 70 °C (decomp)

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.45 (m, 1H), 7.12 (m, 1H), 7.04 (dd, *J* = 1.6, 7.7 Hz, 1H), 6.99 (td, *J* = 1.6, 7.7 Hz, 1H), 5.56 (br. s, 1H), 4.38 (t, *J* = 6.1 Hz, 1H), 4.20 – 4.13 (m, 2H), 4.05 – 3.99 (m, 2H), 3.57 (t, *J* = 5.0 Hz, 4H), 3.39 (s, 6H), 2.80 (d, *J* = 6.1 Hz, 2H), 2.24 (s, 6H).

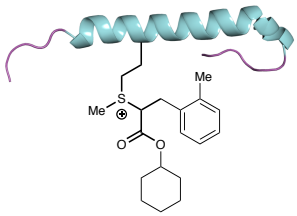
^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.6 (2C), 146.2 (2C), 138.8, 132.7, 132.3, 127.5, 126.4, 125.8, 101.8 (2C), 70.8 (2C), 62.9 (2C), 59.1 (2C), 41.2, 34.3, 19.6 (2C).

IR: 3337, 2923 (br), 2890, 2812, 1687, 1635, 1616, 1486, 1471, 1263, 1200, 1089, 1024, 760, 751.

HRMS-$(ESI)^+$ (m/z) Found (M+H): 484.1140, Calc'd $C_{22}H_{29}{}^{81}BrO_6N$ requires 484.1152.

Rf: 0.3 (50% EtOAc:40-60 Petroleum ether)

Photochemical Alkylation Procedure of Exenatide Derivative 4g



A 5 mL microwave tube was charged with a solution of aqueous, unpurified conjugate **4g** (400 µM, 50 µL). To the resulting solution was added a magnetic stirrer and Hantzsch ester **14b** (2.5 mg, 6.0×10^{-3} mmol, final sol'n conc = 30 mM). The tube was then capped with a septum and subsequently evacuated and refilled with N_2 5 times. To the tube was added added $[Ru(bpy)_3]^{2+}Cl_2 \bullet 6H_2O$ (5 mM in 3:1 CH_3CN:H_2O, freshly sparged w/ N_2, 40 µL, final conc = 1 mM), freshly sparged CH_3CN (70 µl), and freshly sparged H_2O (20 µL) . To this resulting mixture was added $K_2S_2O_8$ (100 mM in H_2O, 20 µL, final conc = 10 mM), and the mixture was immediately** irradiated with a Kessil 40-watt light bulb that was placed at a 5 centimeter distance from the microwave tube for 5 minutes. A constant temperature was maintained by placing the microwave tube under a stream of air over the course of the irradiation. The reaction mixture was then extracted twice with 1:1 ethyl acetate:diethyl ether and the excess volatiles were removed from the aqueous layer using a rotary evaporator. The resulting solution was then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6µm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and the ligation was judged to have proceeded in >95% conversion. The resulting solution was then analyzed directly *via* LC/MS, (C4 column, Gradient: 5-100% B over 5.2 min then hold for 1 min, then 100-5%B over 1 min, 0.2 ml/min). The reaction was repeated 5 times by two different coauthors, and was found to have converted to alkylated conjugate **18a** in an average of 86% (range from 82-90%) over the 5 runs.

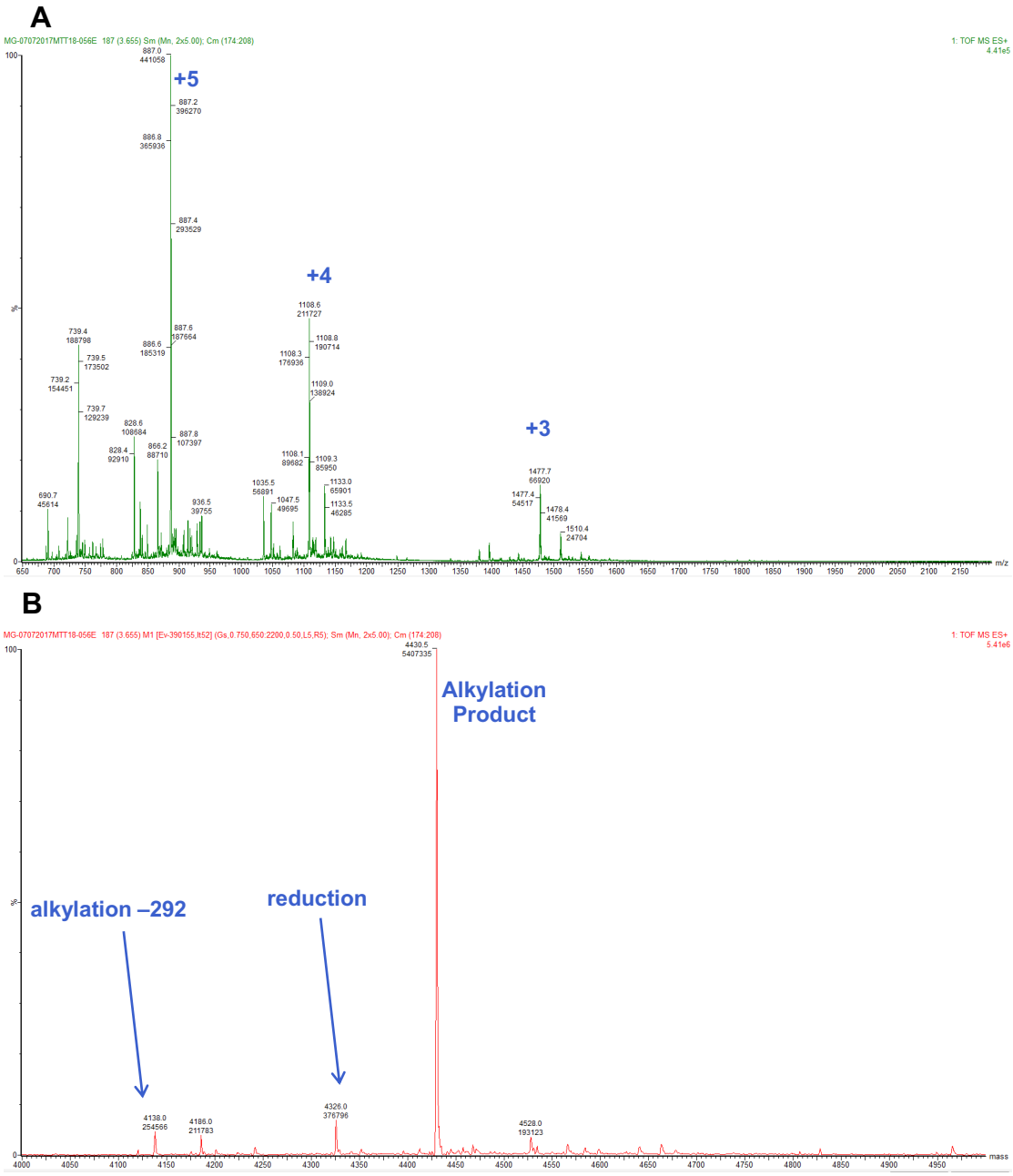


Figure S61. (**A**) Mass chromatogram of the crude reaction mixture of the photoalkylation of exenatide derivative **4g** and Hantzsch ester **14b**. **(B)** Deconvoluted mass chromatogram of the crude reaction mixture.

Isolation of 18a

Isolation of **18a** proceeded as follows: four alkylation reactions were performed in parallel, using the procedure described above on a 400 µL reaction volume scale, a [conjugate] of 125 µM and a 10 minute irradiation time. Following workup, purification was performed *via* reverse-phase HPLC (C18 column (150 x 10 mm, 5 µm), Gradient: 5-95% B over 16 min, then hold for 0.5 min, then 95-5%B over 0.5 min, hold for 2 min, 5 ml/min). The desired fractions were lyophilized to obtain **18a** as a sticky, faint-orange residue that was immediately redissolved in H_2O (200 µL) and stored at –20°C. ICP-MS of the solution containing isolated conjugate **18a** indicated trace Ru levels of less than 1.5 µg/mL.

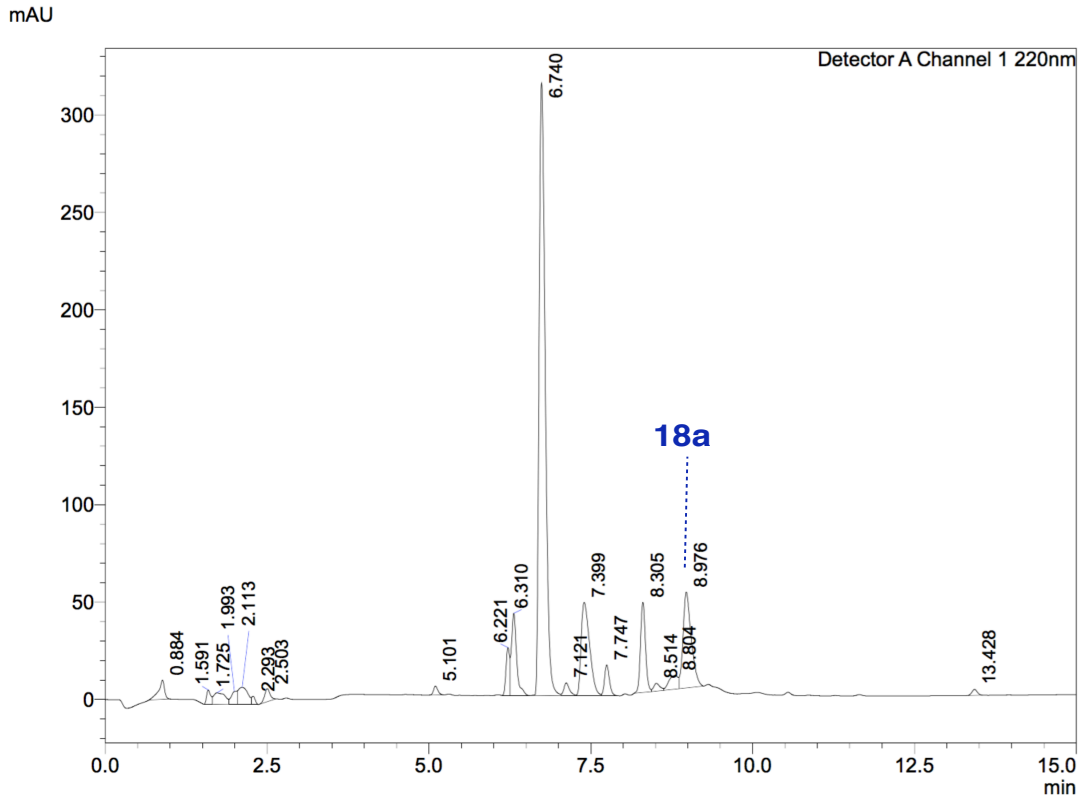


Figure S62. HPLC trace of the crude reaction mixture between **4g** and Hantzsch ester **14b**.

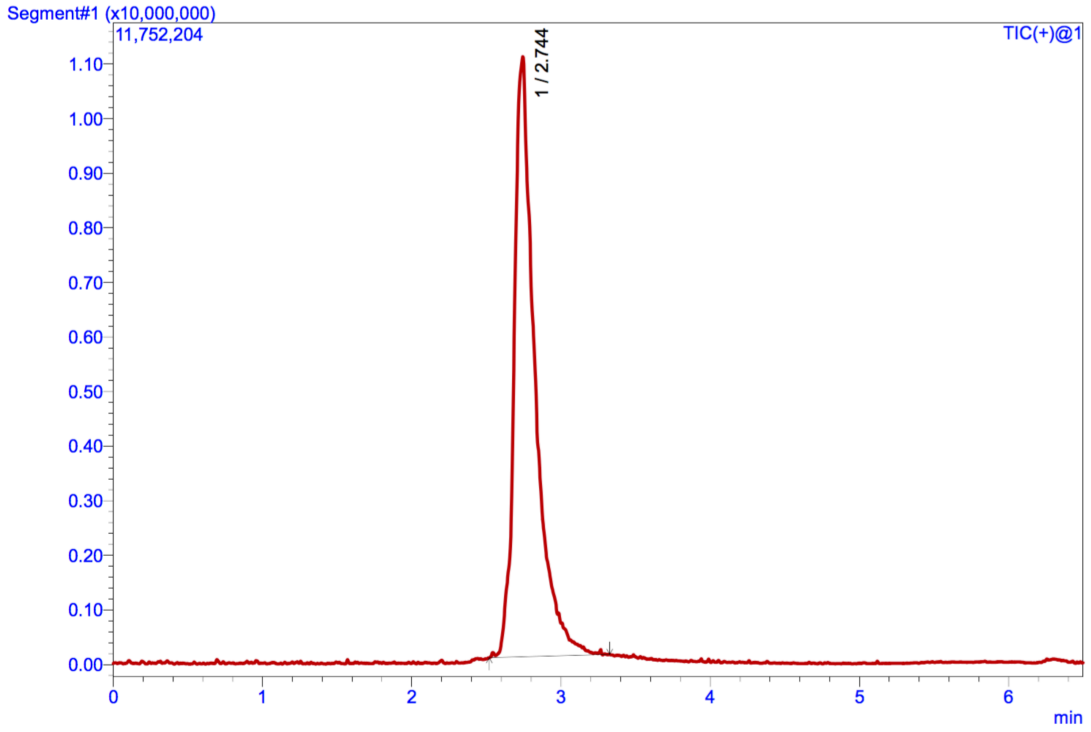


Figure S63. Mass trace of the lyophilized solid **18a**.

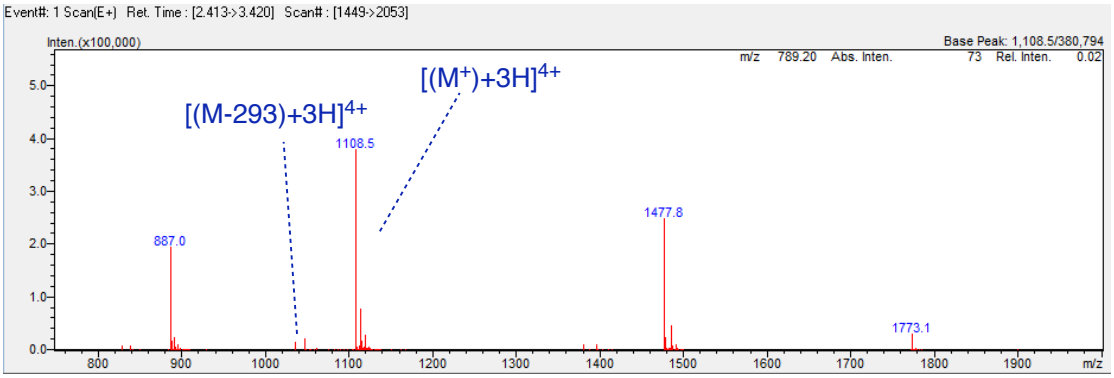


Figure S64. Mass chromatogram of the lyophilized solid **18a**. **18a** was found to contain ~5% free exenatide. Note that **18a** displays the diagnostic fragmentation pattern of cationic sulfur.

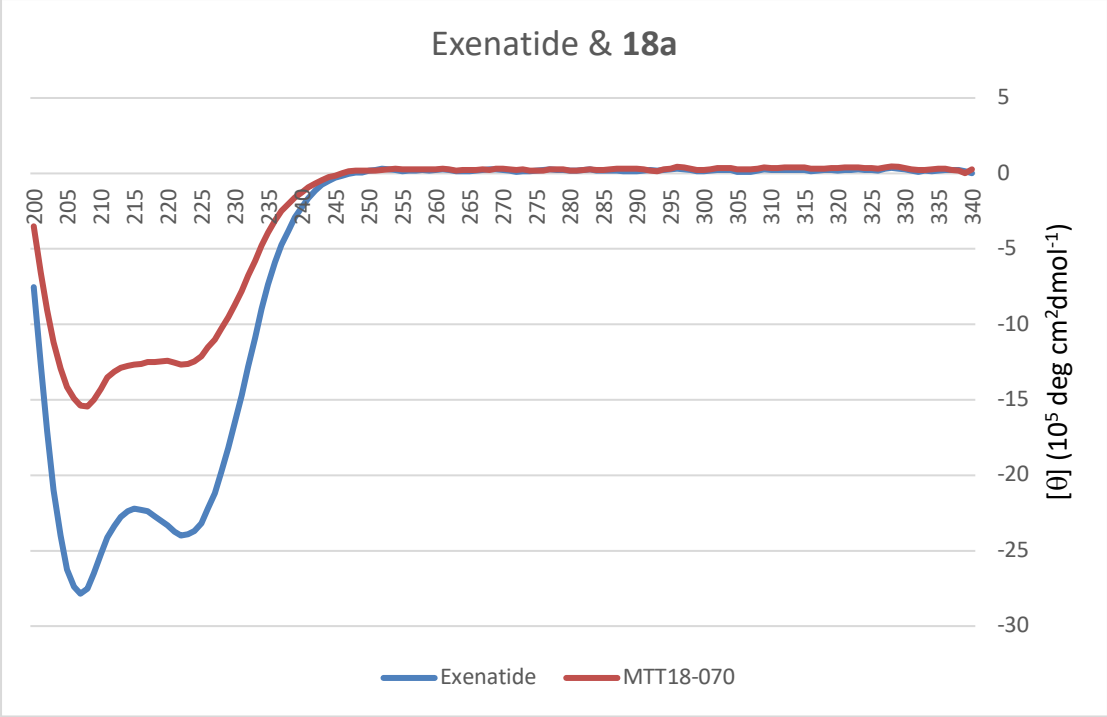
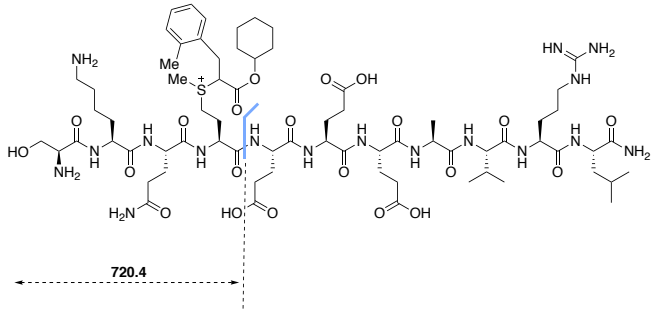


Figure S65. CD spectrum of **18a** (red line) and exenatide (blue line). This spectrum indicates that **18a** retains the structural characteristics of parent exenatide.

Chymotrypsin digest and MSMS analysis of 18a



Variable modifications:
M4 : P172_JN_MTT18 (M)
Ions Score: 49 **Expect:** 9.7e-05
Matches : 7/119 fragment ions using 8 most intense peaks (help)

#	Immon.	b	b^{++}	b*	b^{*++}	b^0	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^0	y^{0++}	#
1	60.0444	88.0393	44.5233			70.0287	35.5180	S							11
2	101.1073	216.1343	108.5708	199.1077	100.0575	198.1237	99.5655	K	1477.7845	739.3959	1460.7580	730.8826	1459.7739	730.3906	10
3	101.0709	344.1928	172.6001	327.1663	164.0868	326.1823	163.5948	Q	1349.6895	675.3484	1332.6630	666.8351	1331.6790	666.3431	9
4	349.2070	720.3875	360.6974	703.3609	352.1841	702.3769	351.6921	M	1221.6310	611.3191	1204.6044	602.8058	1203.6204	602.3138	8
5	102.0550	849.4301	425.2187	832.4035	416.7054	831.4195	416.2134	E	***845.4363***	423.2218	828.4098	414.7085	827.4258	414.2165	7
6	102.0550	978.4727	489.7400	961.4461	481.2267	960.4621	480.7347	E	***716.3937***	358.7005	699.3672	350.1872	698.3832	349.6952	6
7	102.0550	1107.5153	554.2613	1090.4887	545.7480	1089.5047	545.2560	E	***587.3511***	294.1792	570.3246	285.6659	569.3406	285.1739	5
8	44.0495	1178.5524	589.7798	1161.5258	581.2666	1160.5418	580.7745	A	***458.3085***	229.6579	441.2820	221.1446			4
9	72.0808	1277.6208	639.3140	1260.5942	630.8008	1259.6102	630.3088	V	***387.2714***	194.1394	370.2449	185.6261			3
10	129.1135	1433.7219	717.3646	1416.6954	708.8513	1415.7113	708.3593	R	***288.2030***	144.6051	271.1765	136.0919			2
11	86.0964							L	132.1019	66.5546					1

Match to Query 4498: 1562.824056 from(391.713290,4+) intensity(13962992.0000) rtinseconds(2027) scans(11117) index(6222)
Title: File4108 Spectrum6311 scans: 11117
Data file \\prot-filesvr1\data\CORE\RAW_DATA_2017_QE\P172_Jennifer_Nelson\P172_MTT18_chymotrypsin_digest.mgf

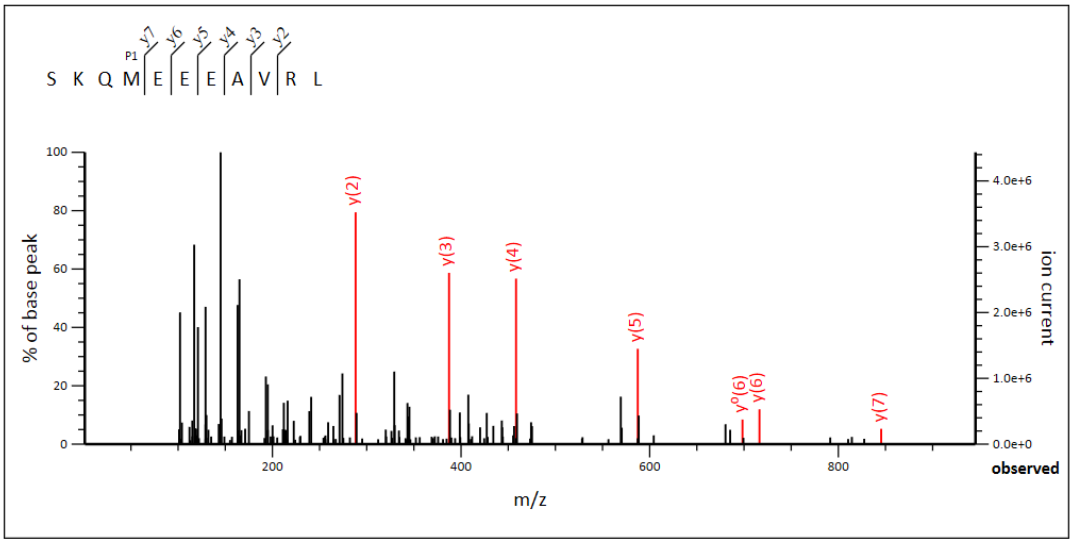
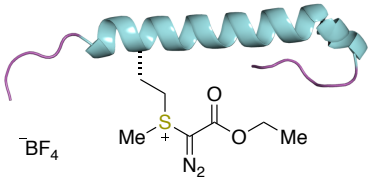
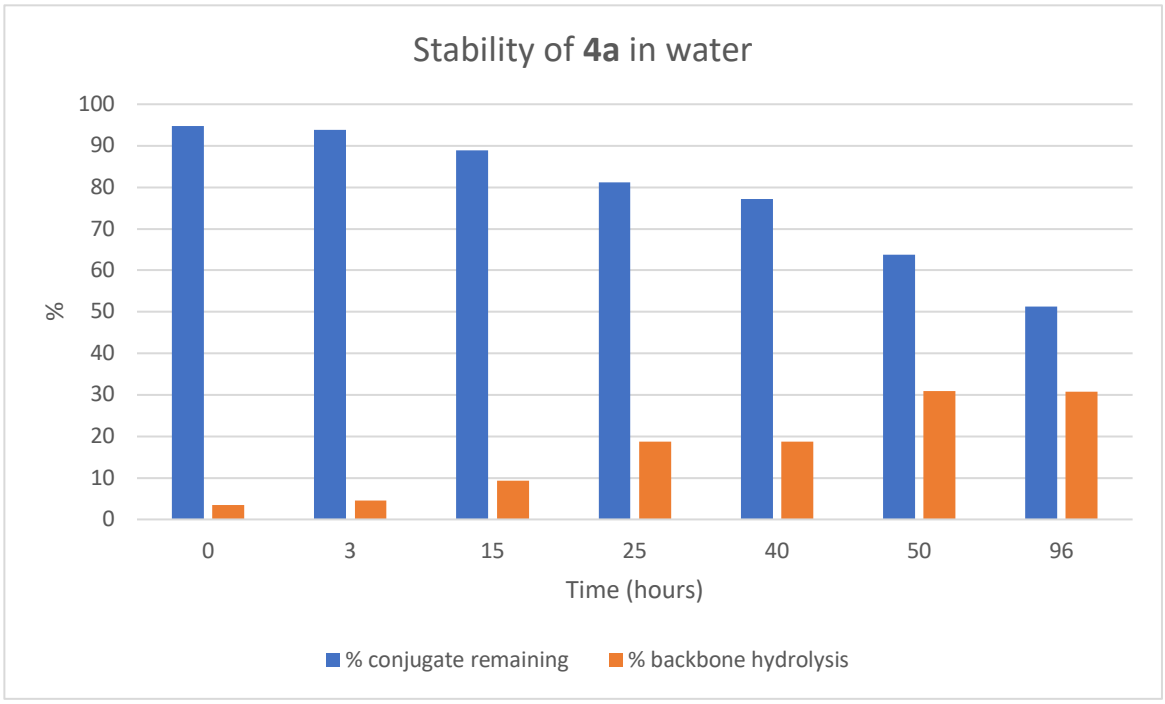


Figure S66: MSMS analysis of **18a**. Chymotrypsin digest fragmented exenatide conjugate **18a** to give a shorter peptide of sequence shown. y_7^+ fragmentation shows the mass of the EEEAVRL fragment to be 845.44 Da. Hence, the remaining peptide fragment is 720.4 Da, confirming the presence of the modification on either of the SKQM residues.

Stability Studies

Stability study of exenatide diazoester conjugate 4a in water





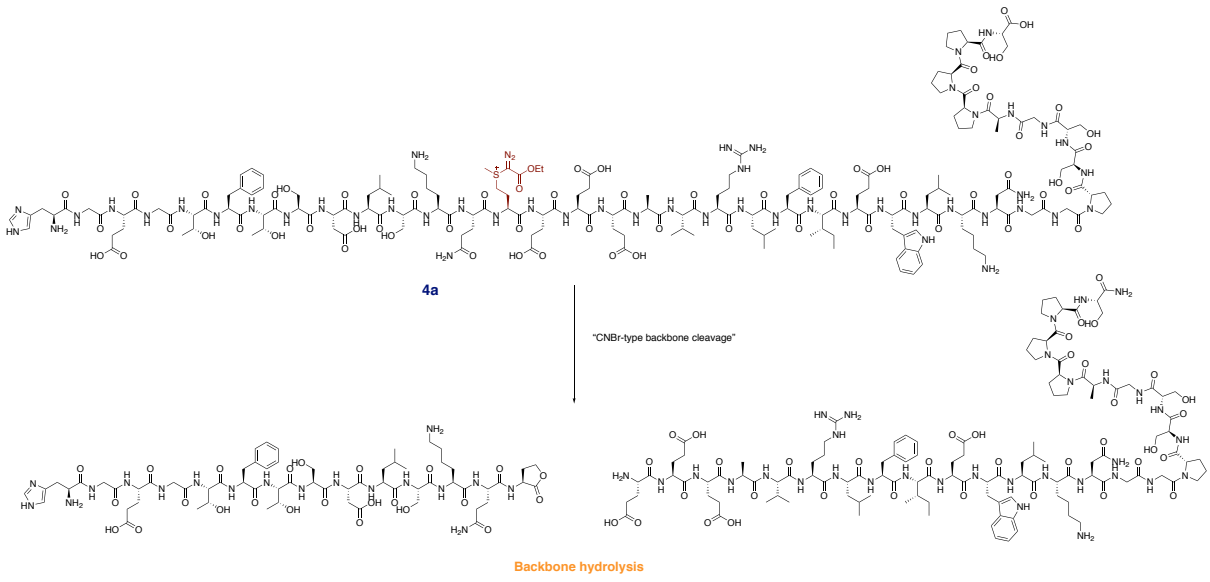
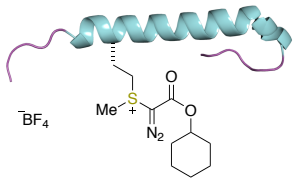
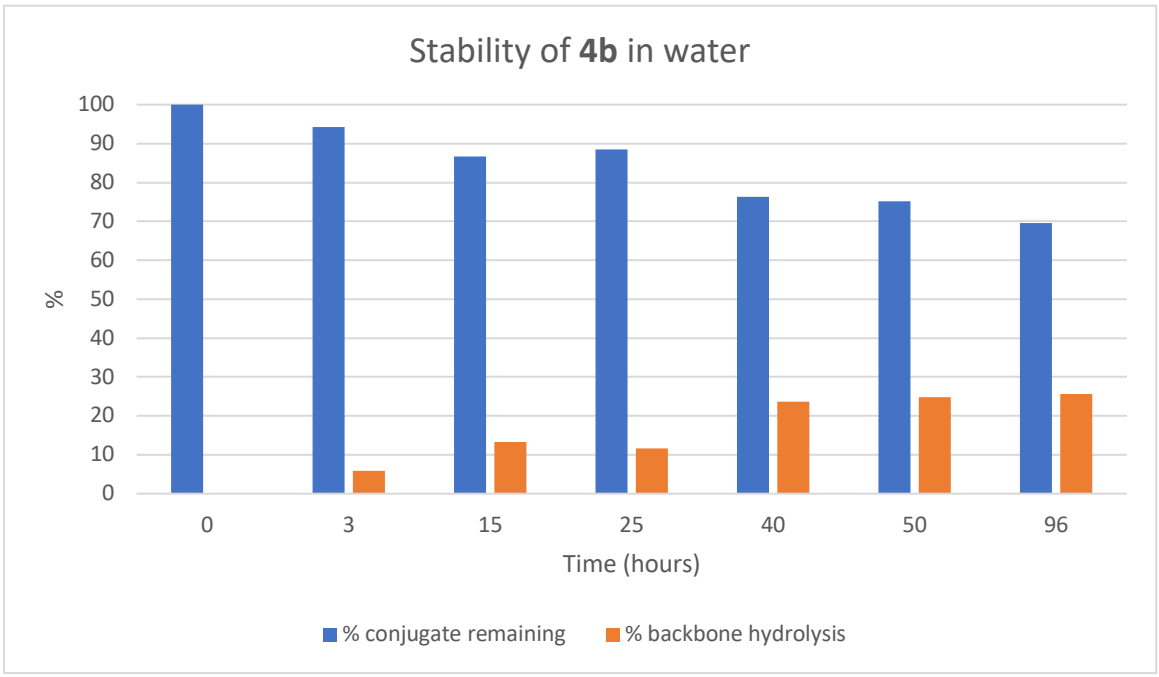


Figure S67: Stability study of **4a** in water, over a period of 96 hours, at room temperature. t = 0 hours begins after storage of **4a** for 24 hours. Degradation occurs predominantly *via* backbone hydrolysis.

Stability of exenatide diazoester conjugate 4b





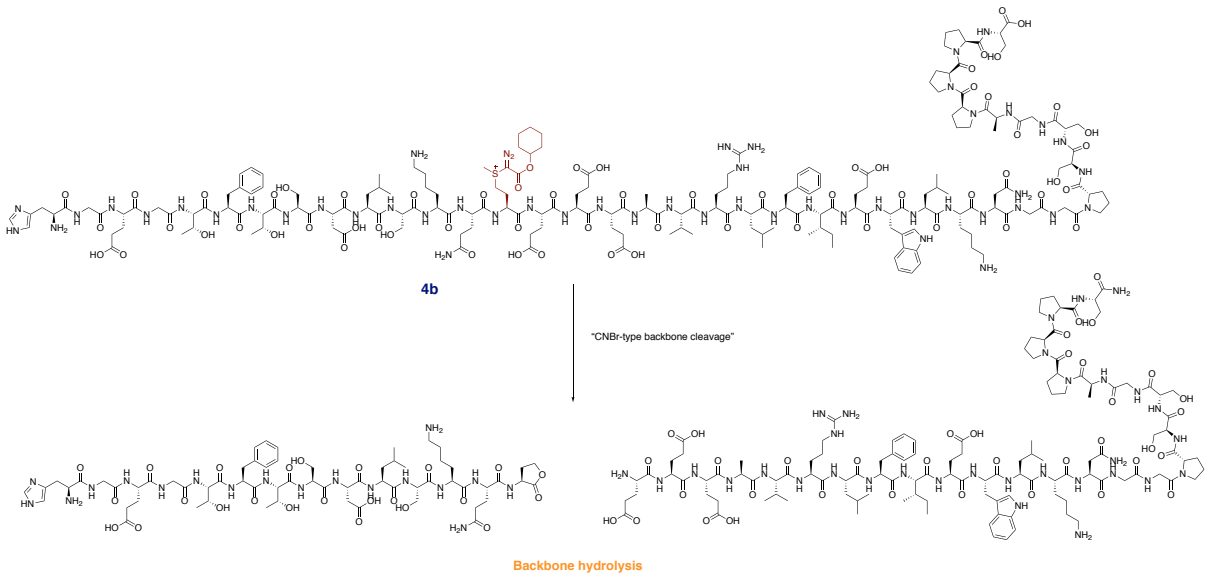
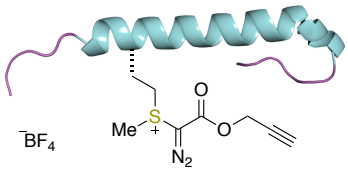
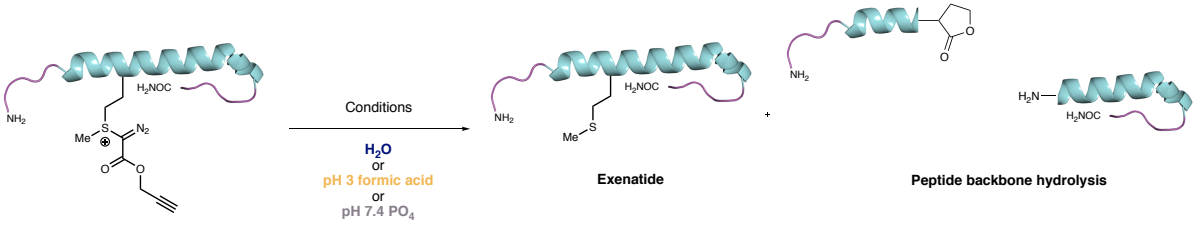


Figure S68: Stability study of **4b** in water, over a period of 96 hours, at room temperature. Degradation occurs predominantly *via* backbone hydrolysis.

Stability study of exenatide diazoester conjugate 4f



A general procedure used to analyze the stability of **4f** is as follows: To a solution of **4f** was added the solution containing the desired additive (final [**4f**] = 100 µM), and was allowed to stand at room temperature. The solution was analyzed directly *via* LC/MS over the time course of the stability study.



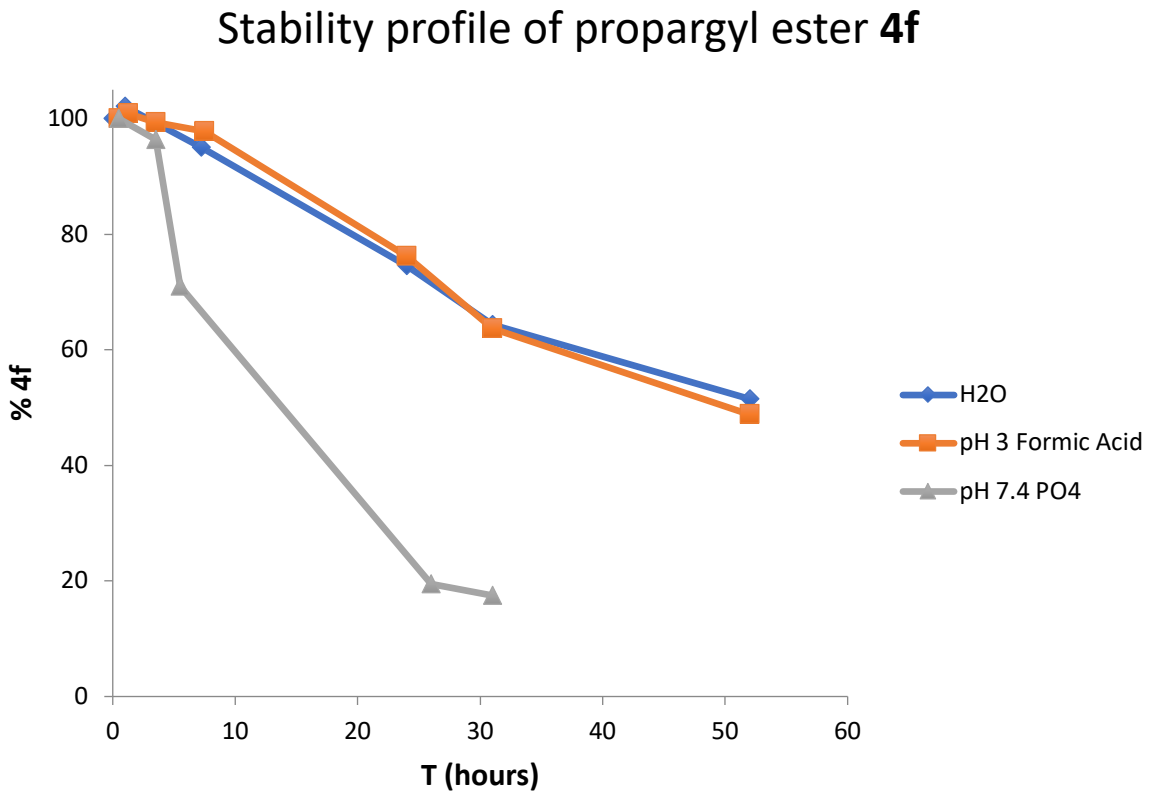
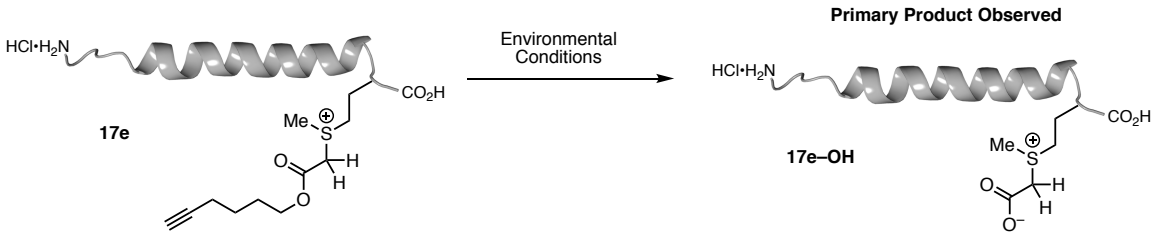


Figure S69: Stability profile of **4f**. For each experiment, **4f** (100 µM) was allowed to stand in solution at room temperature (21°C) over the time course and analyzed by LCMS. **4f** degrades *via* a mixture of peptide backbone hydrolysis and label hydrolysis.

When we exposed **4f** to aqueous solutions of glutathione at 1 mM and 5 mM concentrations, the conjugate displayed partial stability over 8 hours. The primary reaction involved reduction of the diazo motif to the hydrazone. Although there is a chemical change, the methionine

residue remains labeled. Small amounts of peptide cleavage could also be observed but were not quantified. While these results highlight that the methionine conjugate is not stable for extended periods to GSH, it is notable that the label is not cleaved from the substrate. Tailoring the structure of the ester payload may enable us to influence the stability and studies towards this goal are ongoing. We note that the trialkylsulfonium variant of this conjugate (**17f**, **Figure S72**) is stable to glutathione.

Stability of glucagon trialkylsulfonium conjugate 17e



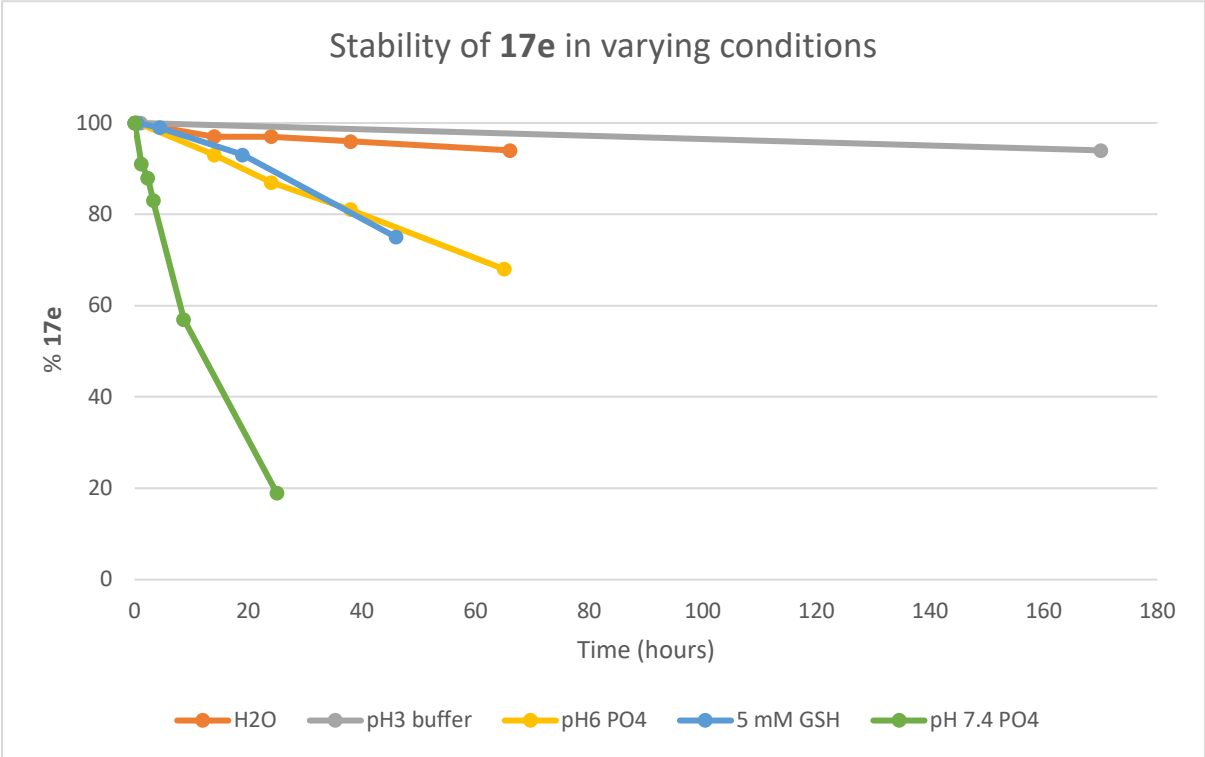
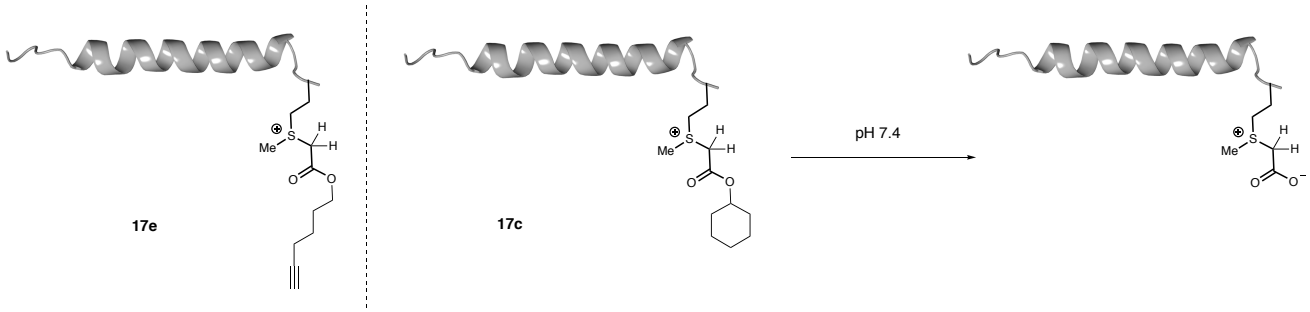


Figure S70: The stability profile of **17e.** For each experiment, **17e** (100 µM) was allowed to stand in solution at room temperature (21°C) over the time course and analyzed by LCMS. In all instances, degradation of **17e** occurs *via* the hydrolysis of the ester moiety to yield a free carboxylate.

Stability of glucagon trialkylsulfonium conjugate 17c



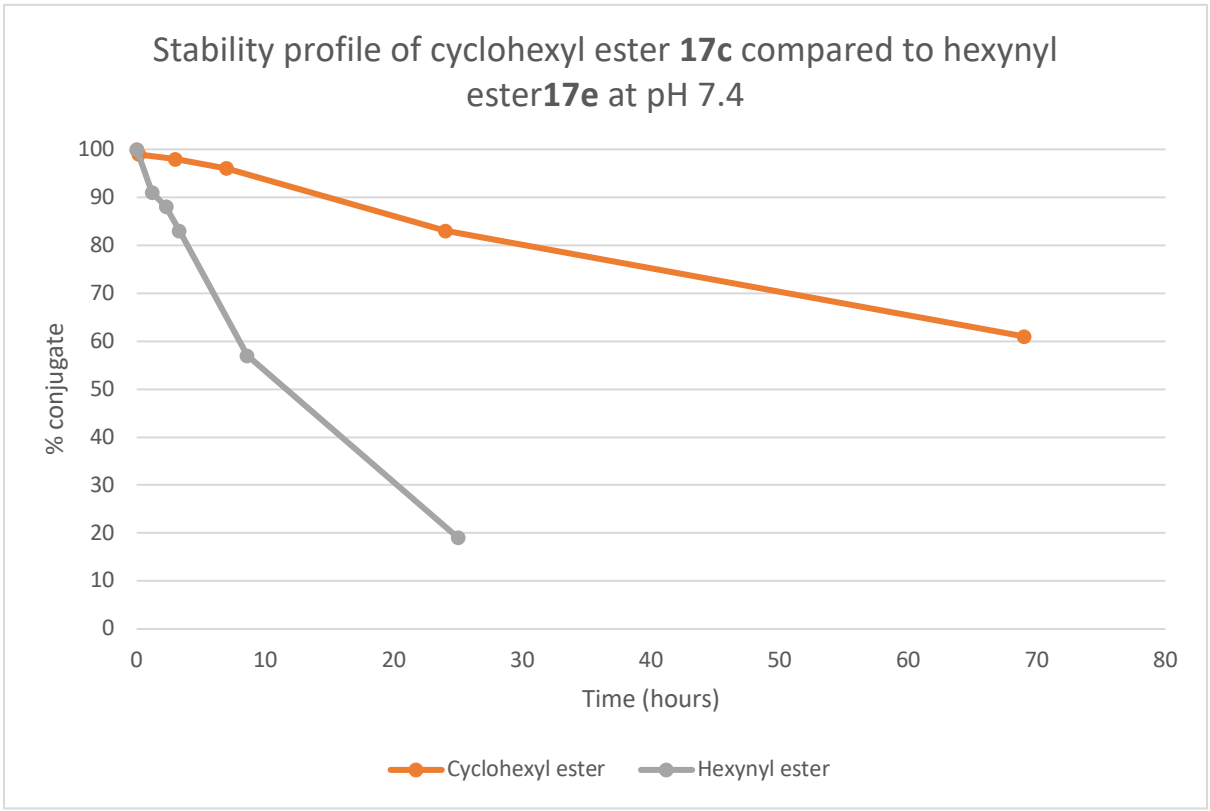
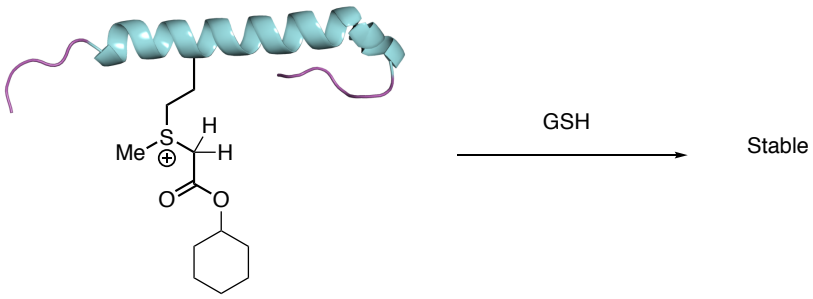


Figure S71: Stability profile of **17c** compared to **17e** at pH 7.4 phosphate buffer. The increased steric hindrance of the cyclohexyl ester significantly increases the half-life of the conjugate, from 10 hours to 90 hours. As with **17c**, **17e** decomposes exclusively *via* hydrolysis of the ester group to yield a free acid.

Stability of exenatide trialkylsulfonium conjugate 17f to glutathione



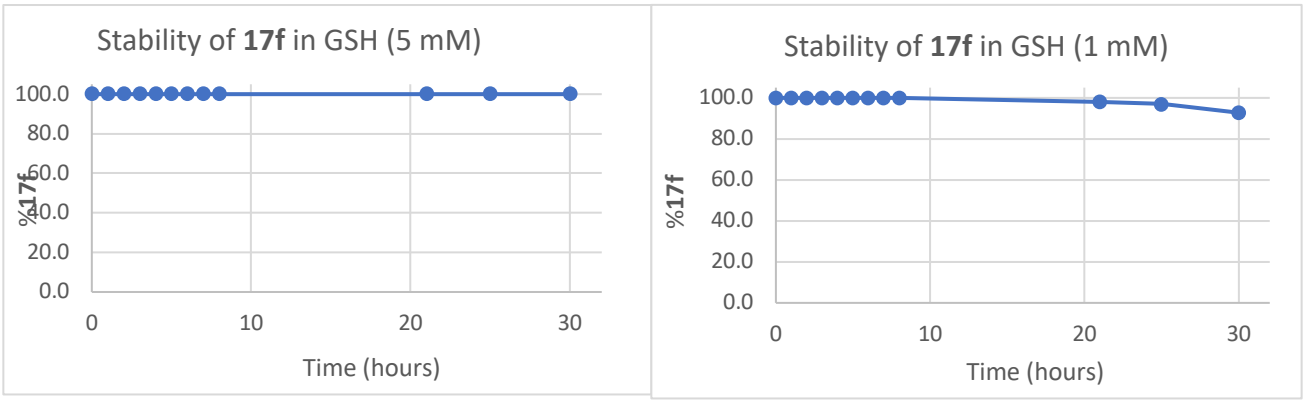


Figure S72: Stability profile of **17f** in the presence of glutathione. Small amount of ester hydrolysis observed (<5%) at t=30 hours.

Mass-Spectral Characteristics of α-sulfonium diazoester-containing protein conjugates

Proteins and peptides that were functionalized in this study were characterized by analyzing their mass chromatograms (positive ionization mode) for a diagnostic fragmentation pattern that is well established with alkylated methionine residues[8-10]. Upon ionization, alkylated methionine residues can fragment at the C_γ-SR_2 bond to produce an $[(M\text{-}SR_2\text{–}H)+H]^+$ ion in addition to the formation of the parent molecular ion $[M]^+$. As this fragmentation pattern is unique to methionine, it can function as a key fingerprint for assigning reactivity at methionine residues. In most of our examples, we used this "sulfonium-fingerprint" as a key diagnostic that the protein modification was indeed occurring at methionine.

The α-sulfoniumdiazoesters generated in this study display this diagnostic fragmentation. This is exemplified by **3b**, which was isolated in >90% purity and characterized by ^{1}H- and ^{13}C-NMR and IR spectroscopy. The ^{1}H NMR clearly shows **3b** as a **single pure product**. ESI-MS of **3b** clearly displays this fragmentation of $[M]^+$ (319.1061 M/Z) and $[(M\text{-}SR_2)+H]^+$ (159.0760 M/Z) (**Figure S73**), showing that this fragment is **created upon ionization in the mass spectrometer** rather than a real species present in the isolated sample of **3b**.

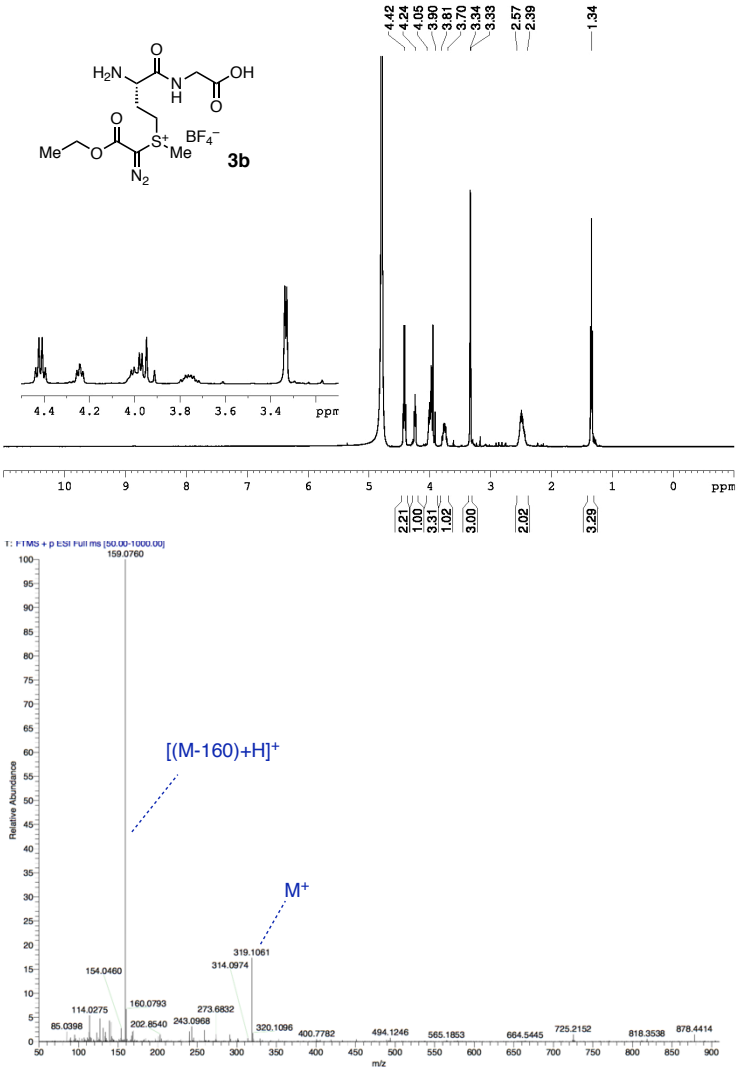


Figure S73: ^{1}H NMR and mass spectrum of purified small molecule diazoester conjugate **3b**

The same fragmentation pattern is observed for the peptide and protein conjugates involved in this study. A number of key experiments differentiate this fragmentation pattern from the possible intramolecular displacement decomposition pathway ("cyanogen bromide type") reactivity.

Compare the two mass traces from **4a** and **4d** (**Figure S74**). Due to the non-polar aliphatic chain, **4d** elutes at a much later retention time (3.80 min) than **4a** (2.75 min) under identical conditions. Yet, in the peaks corresponding to both **4a** and **4d, at differing retention times**, the identical fragmentation pattern is observed (1035.4, 1380.4 etc.). Cyanogen bromide-type cleavage of both these conjugates would yield identical products, which **should have identical retention times**, yet this is not observed.

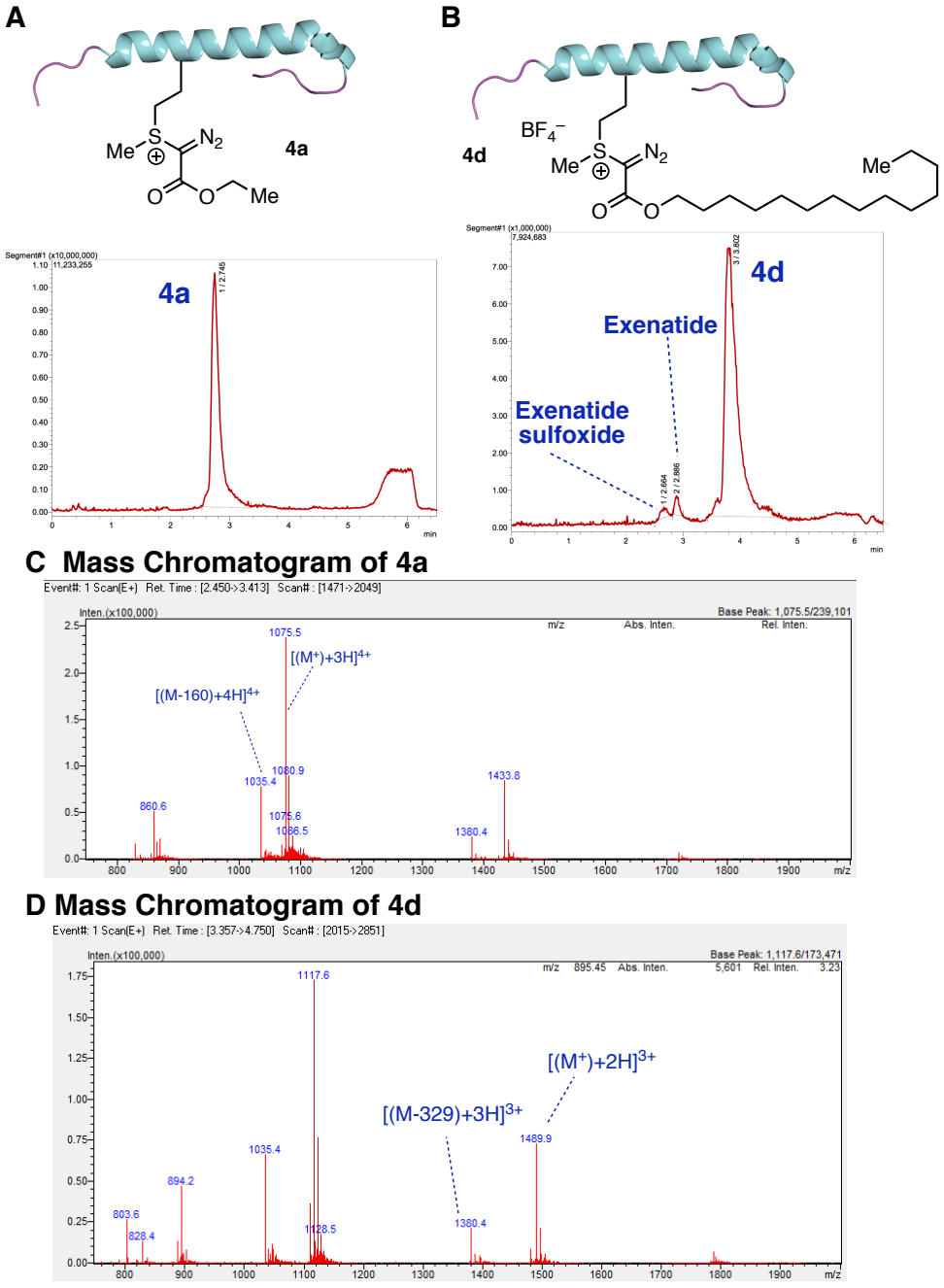


Figure S74: Exenatide ethyl ester **4a** and myristyl ester **4d** conjugates and mass traces

This effect is also clearly illustrated in the stability assays of glucagon conjugate **17c**. Over time, **17c** hydrolyses to form **17c**-OH, as depicted in **Figure S75**. **17c** and **17c**-OH both display the sulfonium fingerprint (**Figure S75-A&B**), and a mass-based search of m/z=859 (**Figure S75-C**), definitively shows that this fragmentation pattern is associated with the two different compounds, at two **different retention times**.

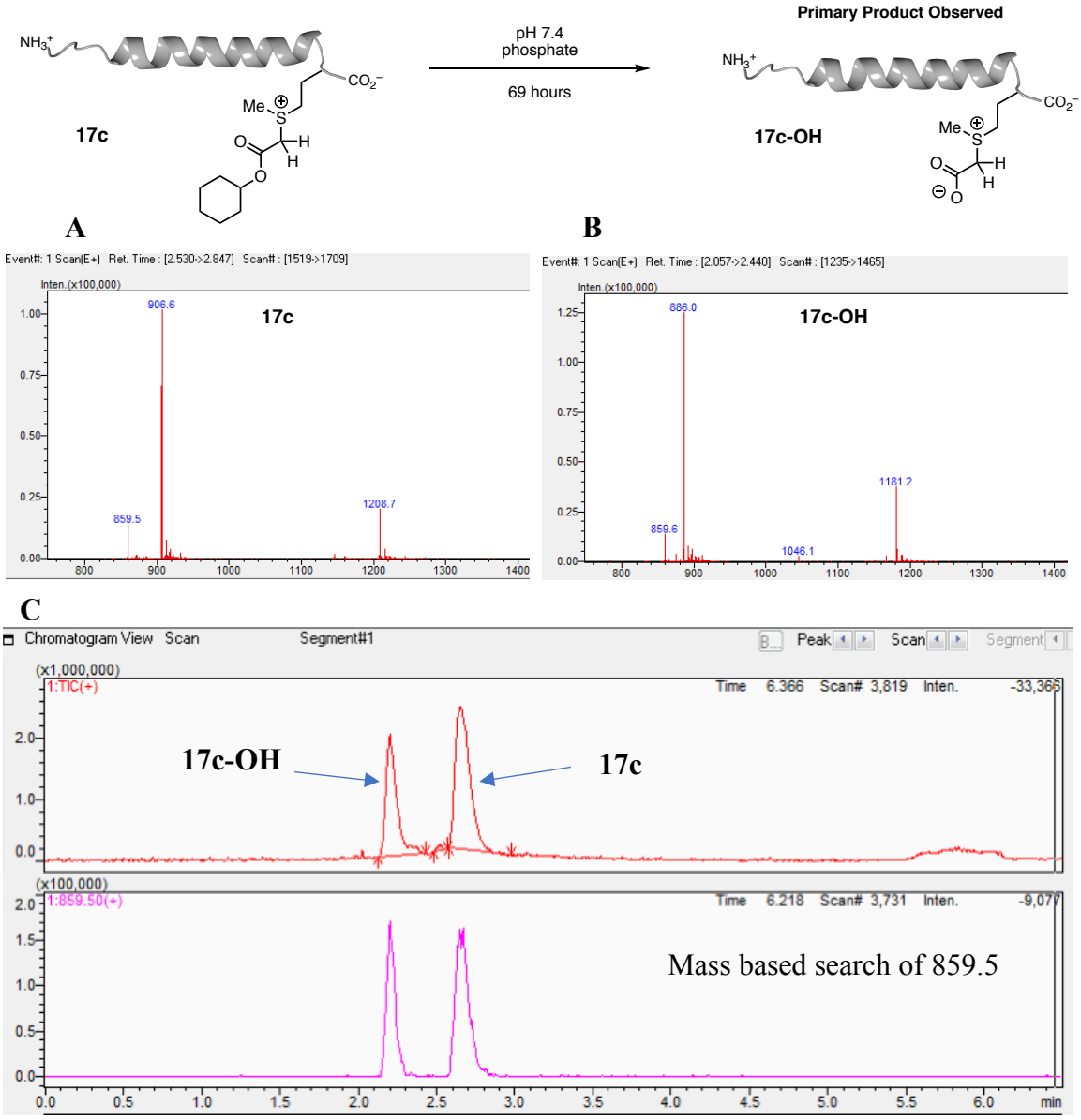


Figure S75: (A) Mass trace of glucagon cyclohexyl conjugat **17c**. **(B)** Mass trace of glucagon hydrolysis product **17c-OH**. **(C)** Mass based search of fragment ion (MW = 859.5), showing the fragment ion is present in both compounds **17c** and **17c-OH**, both of which elute at different retention times.

Finally, reducing the α-sulfoniumdiazoester **5** followed by subsequent hydrolysis releases free methionine (**Figure S76**). Upon hydrolysis, the $[(M\text{-}SR_2)+H]^+$ peak disappears completely. If the $[(M\text{-}SR_2)+H]^+$ peaks were the result of iminolactone formation, then it would be expected that these peaks either 1) **should not be affected by the reduction** or 2) should **hydrolyze** to form subsequent lactone and free amine in equally appreciable intensities. Instead, we see complete disappearance of this ion and **reformation of free peptide** in excellent purity,

indicating this ion is merely **formed upon ionization** rather than a species which is present in isolated samples.

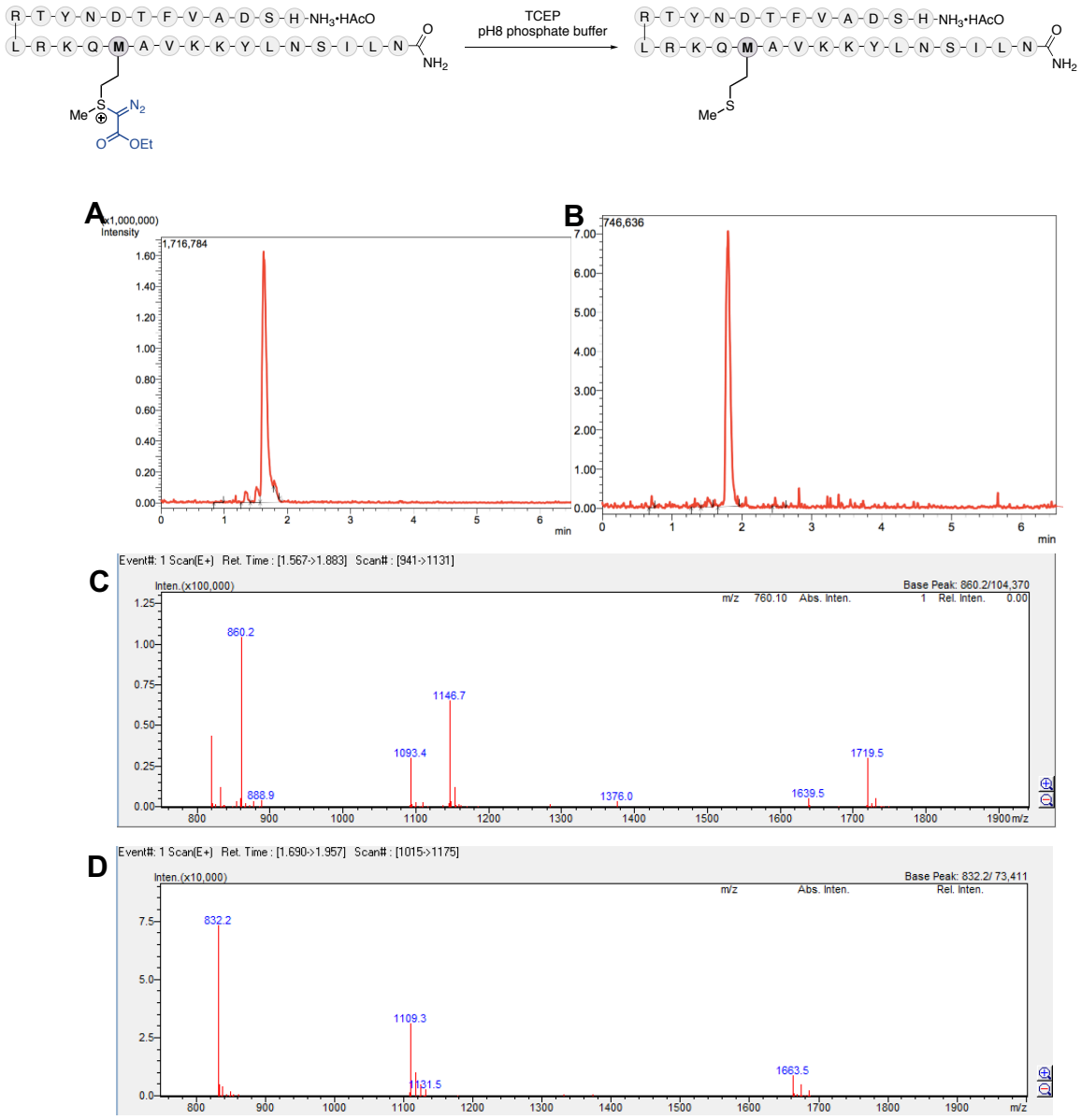


Figure S76. (**A)** Mass trace of **5**. **(B)** Mass trace of the crude mixture between **5** and TCEP at pH 8. **(C)** Mass spectrum of **5** (positive ionization mode, TIC from T_r=1.57-1.88 min.) **(D)** Mass spectrum of the crude mixture between **5** and TCEP at pH 8 (positive ionization mode, TIC from T_r=1.69-1.96 min.) showing full conversion to free aviptadil.

Similarly, this can be observed in the case of Teriparatide in which two methionine residues are labelled. Fragment ions for the $[(M\text{-}SR_2)+H]^+$ and $[(M\text{-}2(SR_2))+H]^+$ are observed (**Figure S77**). Both of these **ions disappear on reduction** with TCEP, with only free teriparatide observed. If these ions were due to iminolactone, they would still be present after reduction, or would be present as lactone and free amine peptide fragments which would be observed in the mass spectrum.

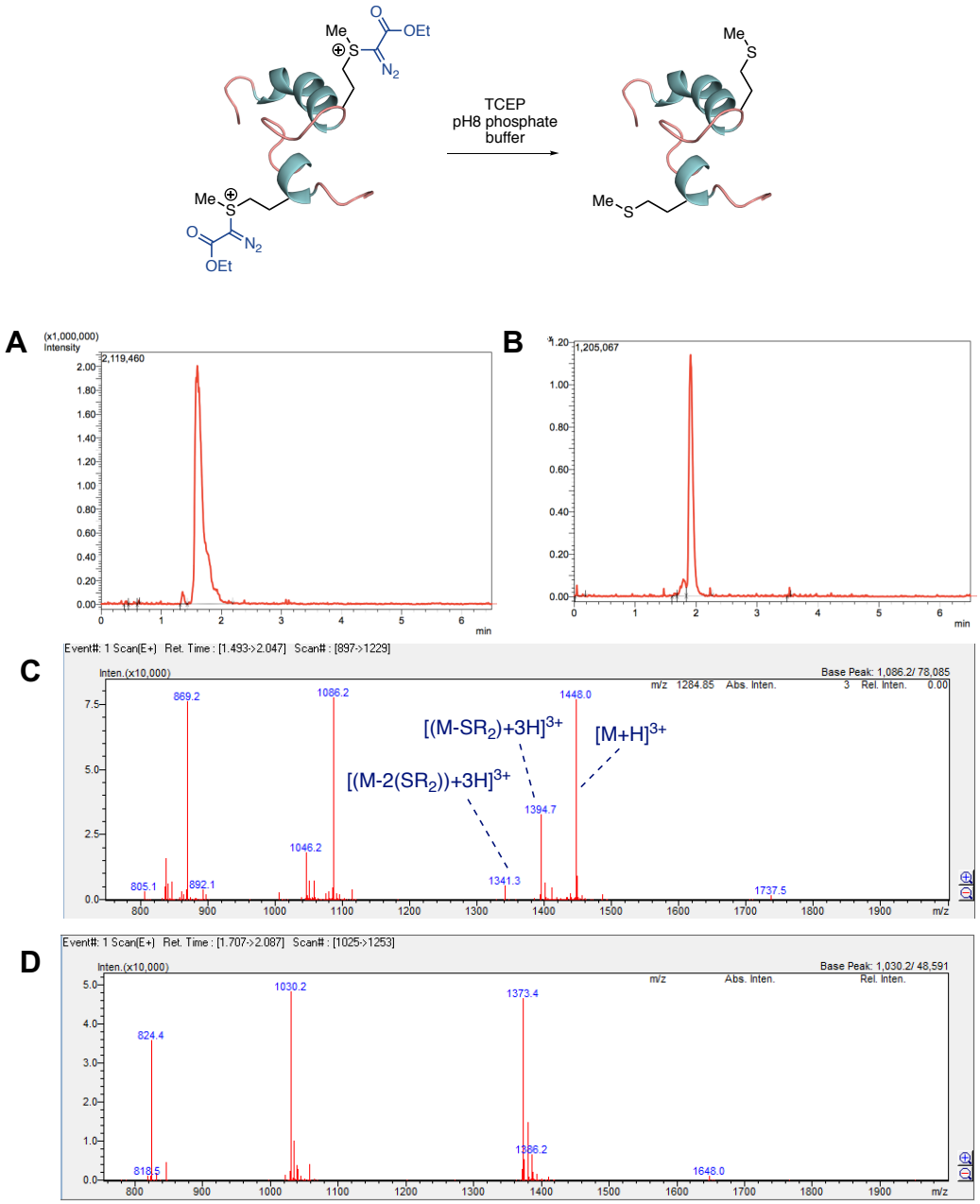


Figure S77. (**A)** Mass trace of **8**. **(B)** Mass trace of the crude mixture between **8** and TCEP at pH 8. **(C)** Mass spectrum of **8** (positive ionization mode, TIC from T_r=1.49-2.05 min.) **(D)** Mass spectrum of the crude mixture between **8** and TCEP at pH 8 (positive ionization mode, TIC from T_r=1.71-2.09 min.) showing full conversion to free teriparatide.

The same affect was observed with Ubiquitin (**Figure S78**). After methionine labelling, Ubiquitin conjugate **11** (8678 Da) displays a dramatic fragmentation upon ionization, wherein the major peak observed is the $[(M\text{-}SR_2)\text{-}H]^+$ mass peak (8517 Da). Upon treatment with 30 mM TCEP in pH 8 phosphate buffer, **11** is reverted back to free Ubiquitin (8565 Da) in >90% conversion, and **no elimination products/hydrolysis of the peptide backbone are observed**.

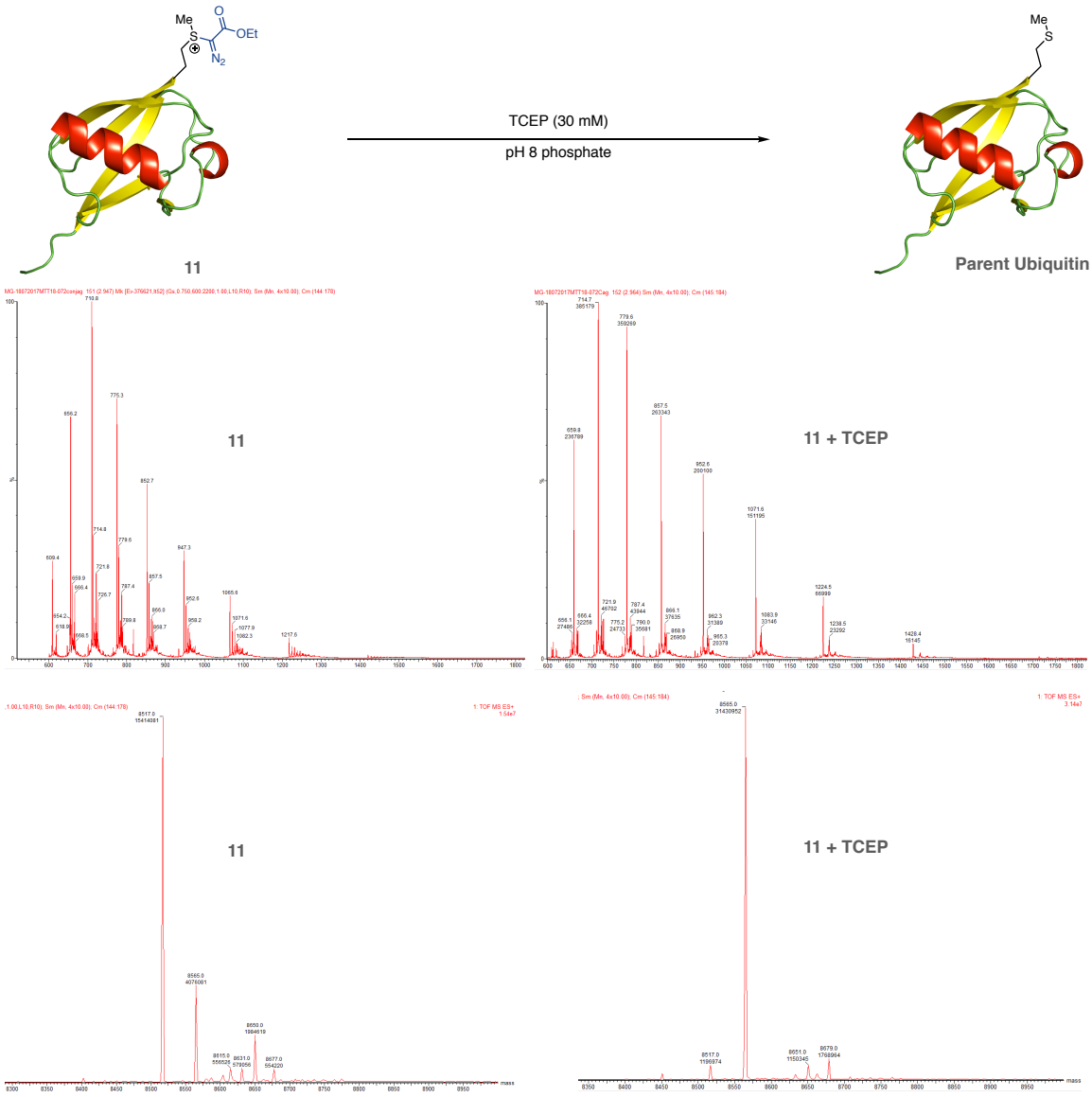
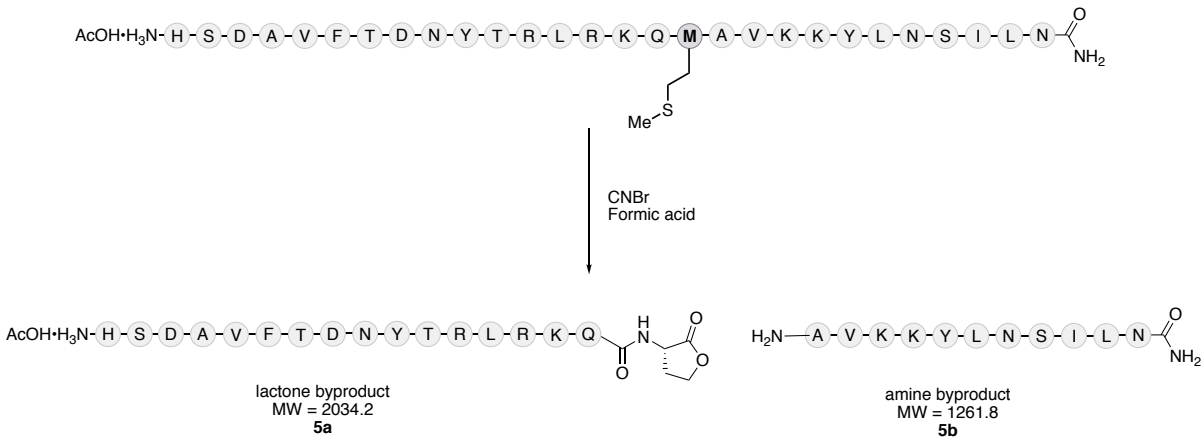


Figure S78: Mass chromatogram and deconvoluted mass spectra of ubiquitin diazoester conjugate **11** and ubiquitin, showing the disappearance of the fragment [M-SR_2] ion on reduction

In summary, the sulfonium-protein conjugates generated in this report display fragmentation patterns upon mass-spectral ionization that is typical of alkyl-sulfonium species and thus provides a key diagnostic fingerprint for site-selectivity of methionine functionalization. 'Cyanogen bromide-type' backbone cleavage (to homoserine lactone and amine peptide fragments) was only observed as a degradation pathway after samples were left for multiple hours at room temperature.

Cyanogen bromide mediated backbone cleavage of Aviptadil



Aviptadil (1.1 mg, 0.33 μmol) was added to a 2 mL vial equipped with a magnetic stirrer. To the vial was added a solution of cyanogen bromide (8.5 mg, 83 μmol) in formic acid (200 μL). The resulting solution was stirred at room temperature for 2 hours. The solution was diluted with water then analyzed directly *via* LC/MS (C18 column (50 x 2.1 mm, 2.6μm), Gradient: 5-95% B over 5 min then hold for 0.5 min, 0.7 ml/min), and was judged to have proceeded in >95% conversion to cleavage products **5a** and **5b**.

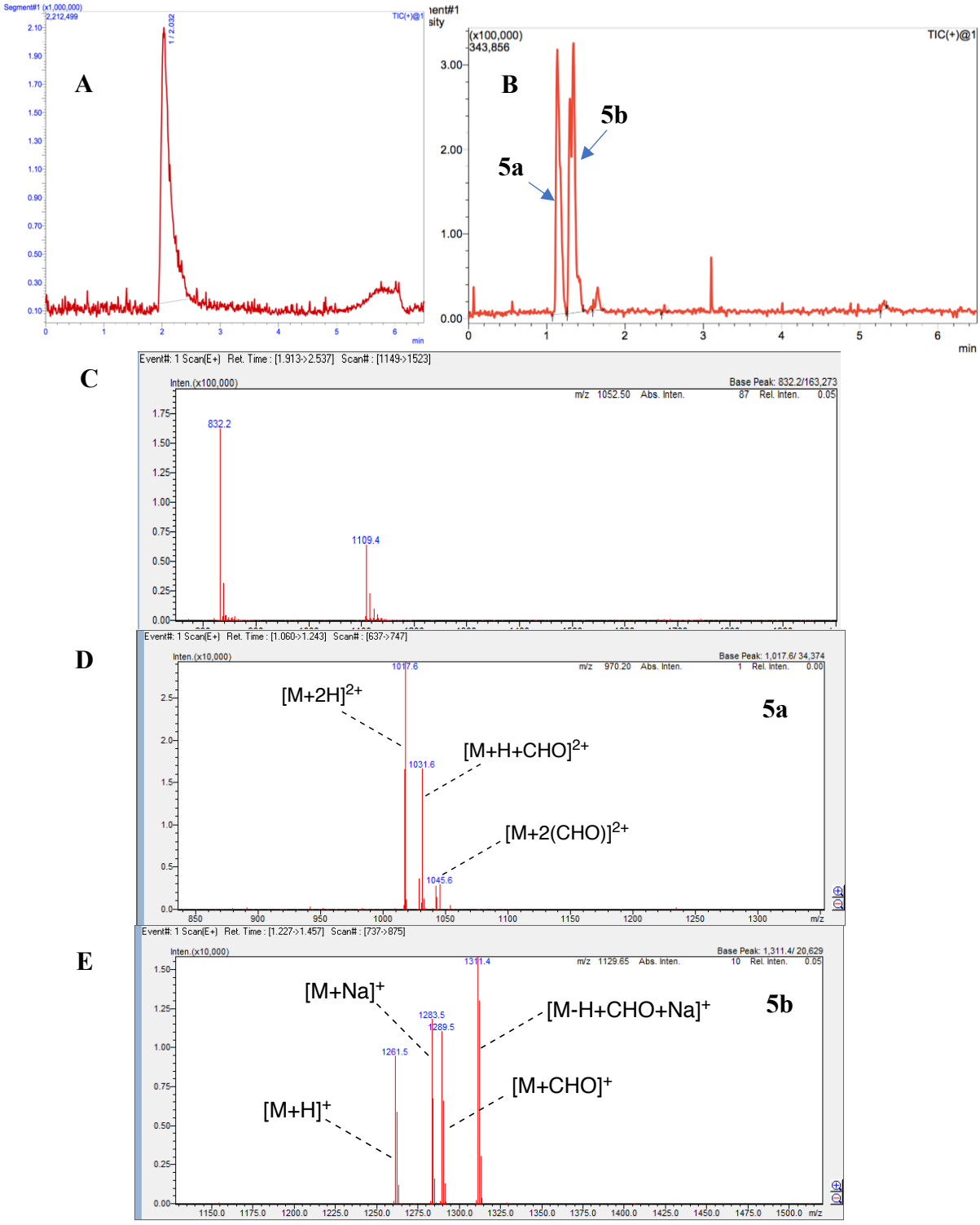
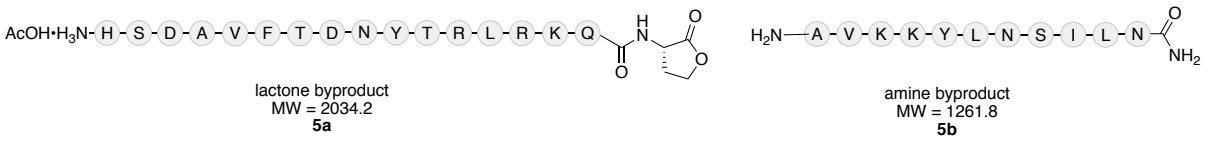


Figure S79: (**A**) Mass trace of Aviptadil (**B**) Mass trace of crude reaction between Aviptadil with CNBr. (**C**) Mass spectrum of Aviptadil. **(D)** Mass spectrum of crude reaction mixture, showing peak 1 (positive ionization mode, TIC from T_r=1.060-1.243 min.) **(E)** Mass spectrum of crude reaction mixture, showing peak 2 (positive ionization mode, TIC from T_r=1.227-1.457 min.)

Mass-based search for products resulting from 'cyanogen bromide-type' backbone cleavage

Cyanogen bromide-type cleavage of the peptide backbone of aviptadil would give resulting lactone and amine-derived byproducts **5a** and **5b**. A mass based search of these byproducts (amine byproduct **5b** $[M+H]^+$ = 1261.8 and lactone byproduct **5a** $[M+2H]^{2+}$ = 1017.8) on the crude reaction mixture showed no evidence of the presence of these backbone hydrolysis products (**Figure S80**).



lactone byproduct
MW = 2034.2
5a

amine byproduct
MW = 1261.8
5b

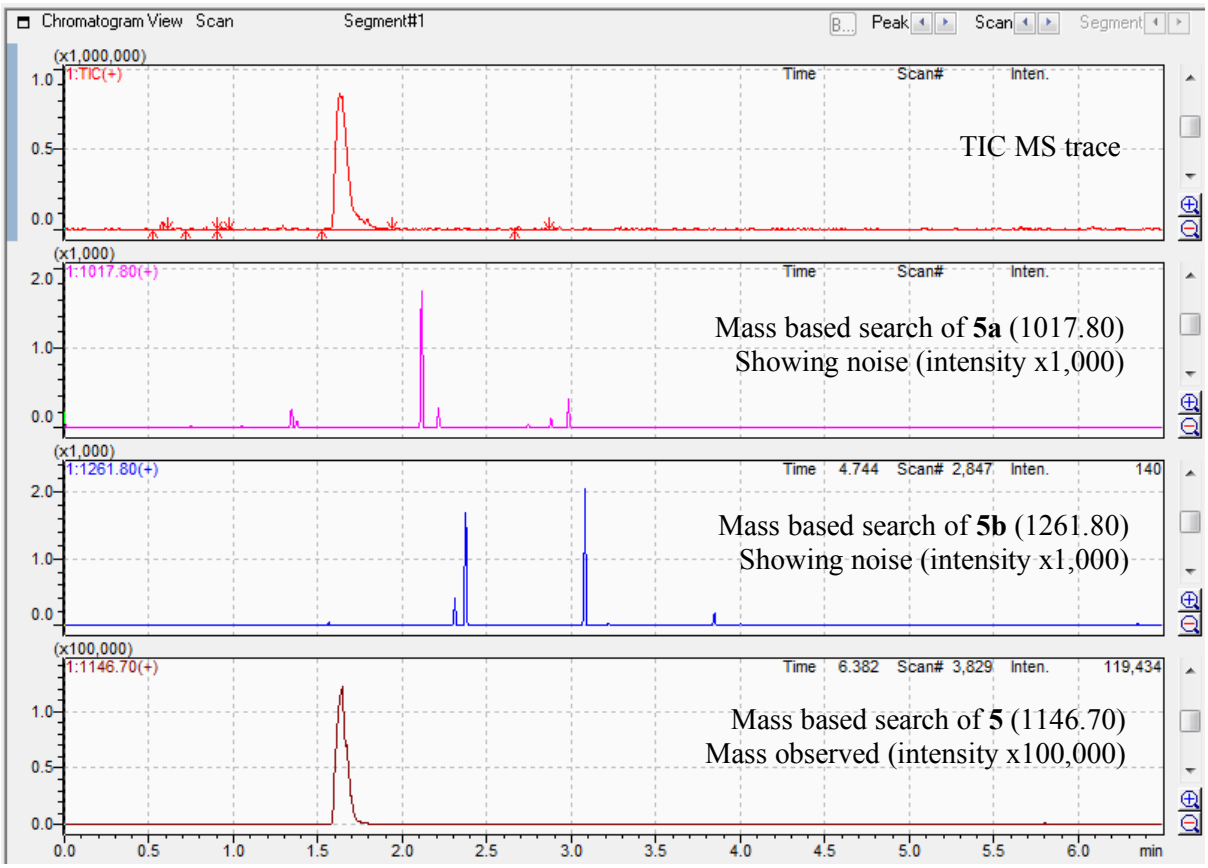


Figure S80: Mass based search on mass trace of crude reaction to form aviptadil conjugate **5**. Search for backbone hydrolysis products (amine byproduct **5b** $[M+H]^+$ = 1261.8 and lactone byproduct **5a** $[M+2H]^{2+}$ = 1017.8) shows only noise, whilst search for one of the product ions of **5** ($[(M^+)+2H]^{3+}$ = 1146.7) clearly shows the presence of aviptadil conjugate **5**.

Aviptadil conjugate **5** was then subjected to reverse phase HPLC purification and subsequent lyophilisation overnight to yield an isolated sample of **5**. A mass based search of the products of cyanogen bromide-type cleavage of the peptide backbone (amine byproduct **5b** $[M+H]^+$ = 1261.8 and lactone byproduct **5a** $[M+2H]^{2+}$ = 1017.8) on the isolated sample of **5** showed a small amount of the presence of these backbone hydrolysis products (**Figure S81**).

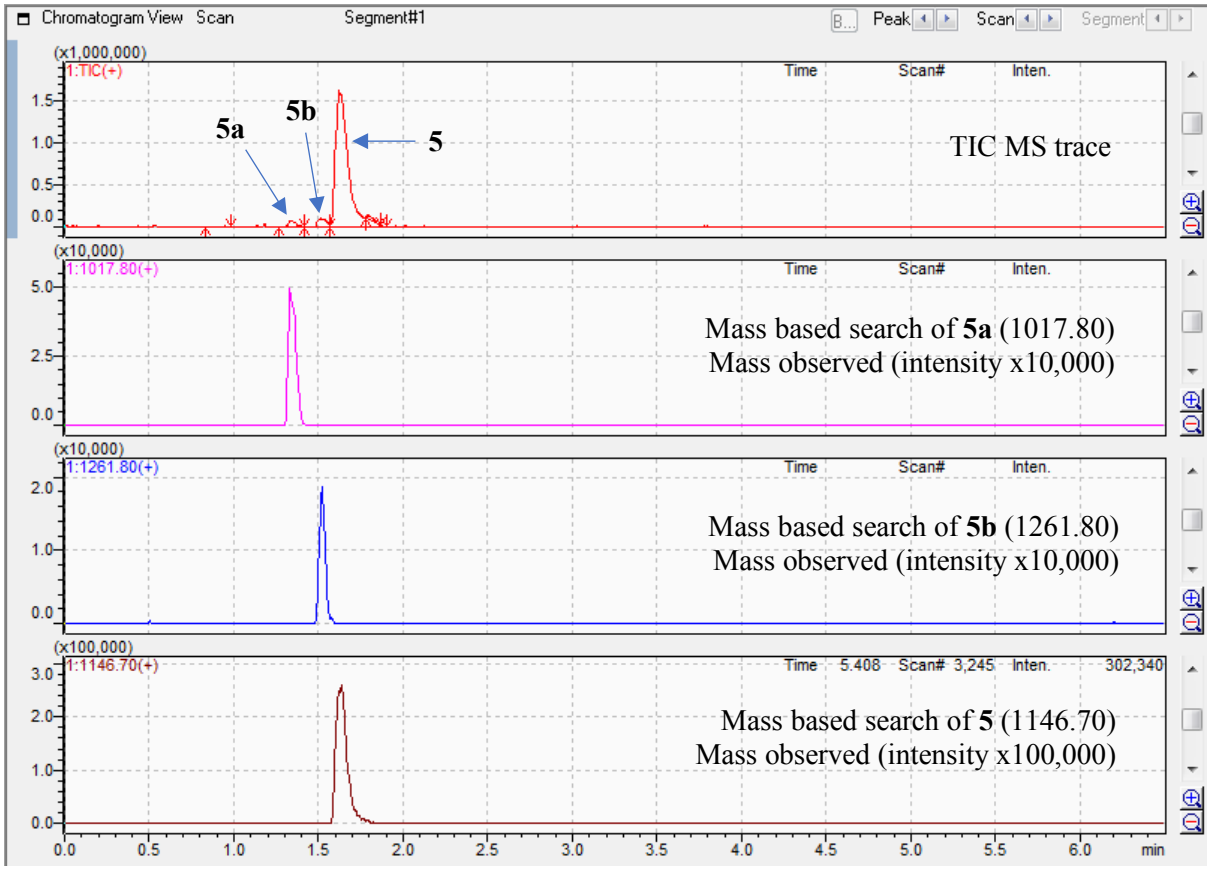
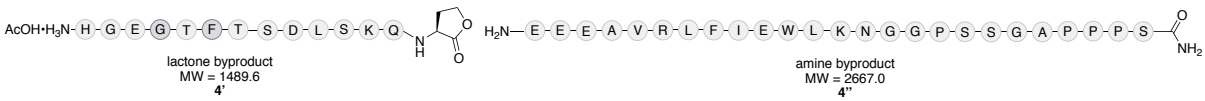


Figure S81: Mass based search on mass trace of isolated aviptadil conjugate **5**. Search for backbone hydrolysis (amine byproduct **5b** $[M+H]^+$ = 1261.8 and lactone byproduct **5a** $[M+2H]^{2+}$ = 1017.8) shows presence of these byproducts. Search for product ion of **5** ($[(M^+)+2H]^{3+}$ = 1146.7) also shows the presence of aviptadil conjugate **5**. Note the difference in intensity of a factor of 10 between backbone hydrolysis byproducts and conjugate **5**, and additionally the differing retention time from the conjugate **5**.

Cyanogen bromide-type cleavage of the peptide backbone of exenatide would give resulting lactone and amine-derived byproducts **4'** and **4''**. A mass based search of these byproducts (amine byproduct **4''** $[M+2H]^{2+}$ = 1334.5 and lactone byproduct **4'** $[M+H]^+$ = 1489.6) on the crude reaction mixture showed no evidence of the presence of these backbone hydrolysis products (**Figure S82**).



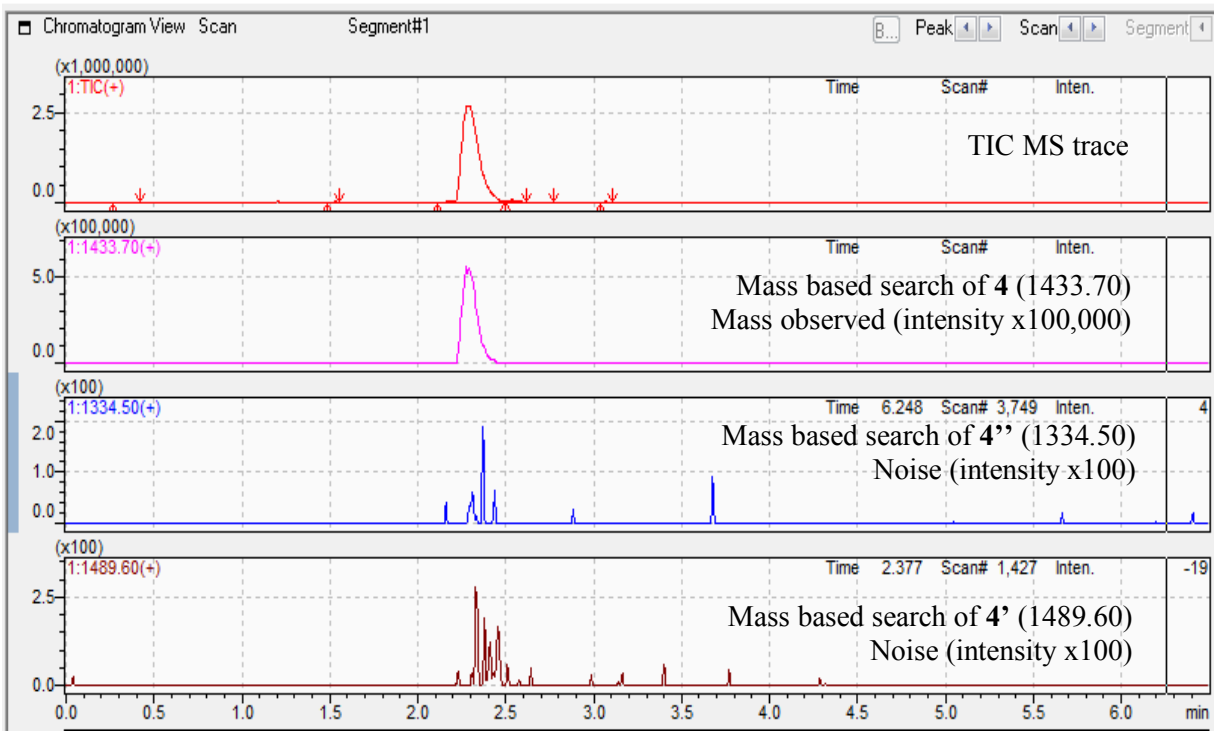


Figure S82: Mass based search on mass trace of crude reaction to form exenatide conjugate **4a**. Search for backbone hydrolysis products (amine byproduct **4''** $[M+2H]^{2+}$ = 1334.5 and lactone byproduct **4'** $[M+H]^{+}$ = 1489.6) shows only noise, whilst search for one of the product ions of **4a** ($[(M^{+})+2H]^{3+}$ = 1433.7) clearly shows the presence of exenatide conjugate **4a**.

In the same manner as the aviptadil conjugate **5**, exenatide conjugate **4a** was then subjected to reverse phase HPLC purification and subsequent lyophilisation overnight to yield an isolated sample of **4a**. A mass based search of the products of cyanogen bromide-type cleavage of the peptide backbone (amine byproduct **4''** $[M+2H]^{2+}$ = 1334.5 and lactone byproduct **4'** $[M+H]^{+}$ = 1489.6) on the isolated sample of **4a** still showed no presence of these backbone hydrolysis products (**Figure S83**). Backbone hydrolysis products in this case are only observed after a number of hours at room temperature (see stability study **Figure S67**).

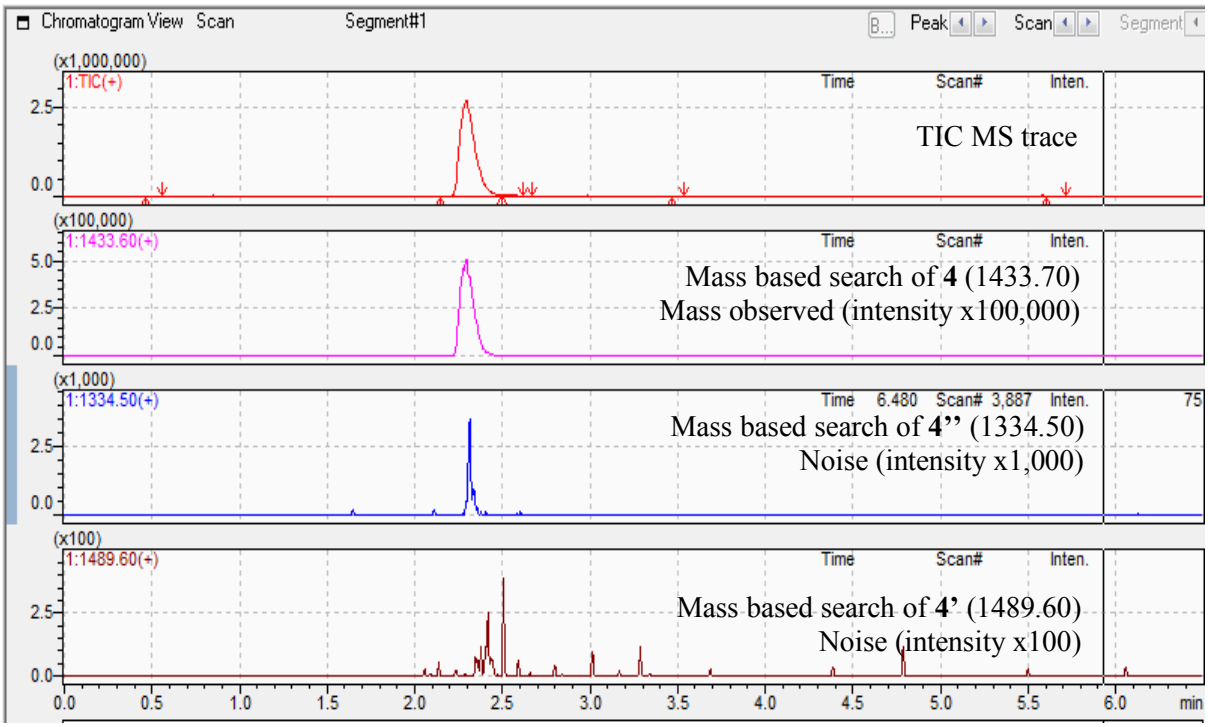


Figure S83: Mass based search on mass trace of isolated exenatide conjugate **4a**. Search for backbone hydrolysis (amine byproduct **4''** $[M+2H]^{2+}$ = 1334.5 and lactone byproduct **4'** $[M+H]^{+}$ = 1489.6) shows no presence of these byproducts. Search for product ion of **4a** ($[(M^{+})+2H]^{3+}$ = 1146.7) shows the presence of exenatide conjugate **5a**

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