N-terminal modification of proteins with *o*-aminophenols

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Supporting Information

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S.1 General methods

Unless otherwise noted, the chemicals and solvents used were of analytical grade and were used as received from commercial sources. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F_{254} plates with visualization by ultraviolet (UV) irradiation at 254 nm and/or potassium permanganate. Purifications by flash chromatography were performed using EM silica gel 60 (230-400 mesh). The eluting system for each purification was determined by TLC analysis. Chromatography solvents were used without distillation. All organic solvents were removed under reduced pressure using a rotary evaporator. Water (ddH_2O) used as reaction solvent was deionized using a Barnstead NANOpure purification system (ThermoFisher, Waltham, MA). Centrifugations were performed with an Eppendorf Mini Spin Plus (Eppendorf, Hauppauge, NY).

S.2 Instrumentation and sample analysis

NMR. ^{1}H and ^{13}C spectra were measured with a Bruker AVB-400 (400 MHz, 100 MHz) or a Bruker AV-600 (600 MHz, 150 MHz) spectrometer, as noted. ^{1}H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 7.26, singlet) or CD_3CN (δ 1.94, quintet). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), br (broad) or m (multiplet). Coupling constants are reported as a J value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.2, triplet) or CD_3CN (δ 118.26, singlet).

Mass Spectrometry. High-resolution electrospray ionization (ESI) and liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) mass spectra were obtained at the UC Berkeley QB3/Chemistry Mass Spectrometry Facility. Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA) and data were analyzed using Data Explorer software. Peptide samples were co-crystallized with α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile (MeCN) to H_2O with 0.1% trifluoroacetic acid (TFA). Synthetic peptides and protein bioconjugates were analyzed using an Agilent 1200 series liquid chromatograph (Agilent Technologies, USA) that was connected in-line with an Agilent 6224 Time-of-Flight (TOF) LC/MS system equipped with a Turbospray ion source.

High Performance Liquid Chromatography. HPLC was performed on Agilent 1100 Series HPLC Systems (Agilent Technologies, USA) outfitted with an Agilent 1200 series automatic fraction collector. Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and inline fluorescence detector (FLD). Semi-preparative reverse-phase HPLC of peptides was accomplished using a C18 stationary phase and a H_2O/ MeCN with 0.1% TFA gradient mobile phase.

Gel Analyses. For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus (Bio-Rad, Hercules, CA), using a 10-20% precast linear gradient polyacrylamide gel (Bio-Rad). The sample and electrode buffers were prepared according to Laemmli.[1] All protein electrophoresis samples were heated for 5-10 min at 95 °C in the presence of 1,4-dithiothreitol (DTT) to ensure reduction of disulfide bonds. Gels were run for 75-90 minutes at 120 V to separate the bands. Commercially available markers (Bio-Rad) were applied to at least one lane of each gel for assignment of apparent molecular masses. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA). ImageJ was used to determine the level of modification by optical densitometry.

S.3 Peptide synthesis

General procedure for solid-phase peptide synthesis. Peptides were synthesized using standard Fmoc-based chemistry on Tentagel S-OH resin (Advanced ChemTech, Louisville, KY). The base peptide sequence (ADSWAG) was synthesized on 1 g of resin and split into 20 x 50 mg aliquots for the addition of the final amino acid. The side chain protecting groups used were: Asn(Trt), Cys(Trt), Asp(tBu), Glu(tBu), His(Trt), Lys(Boc), *p*AF(Boc), Gln(Trt), Arg(Pbf), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu). The C-terminal amino acid (10 equiv) was preactivated at 0 °C with 5 equivalents of diisopropylcarbodiimide (DIC) and then coupled to the resin with 0.1 equivalents of *N*,*N*-dimethylaminopyridine (DMAP) as a catalyst. Deprotection of the Fmoc groups was performed with a 20 min incubation in a 20% v/v piperidine in dimethylformamide (DMF) solution. Coupling reactions were carried out using 20 equivalents of amino acid with 10 equivalents of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and 20 equivalents of *N*,*N*-diisopropylethylamine (DIPEA) in DMF for 20 min. Side-chain deprotection was accomplished using a 1-2 h incuba-

tion with a 95:2.5:2.5 ratio of TFA to H_2O to triisopropylsilane (TIPS). Peptides were cleaved from the resin by a 30-45 min incubation with a 100 mM sodium hydroxide solution. The resulting basic solution was neutralized with 100 mM phosphate buffer, pH 6.5. The cleaved peptides were purified using reverse-phase HPLC with a gradient of water/CH_3CN with 0.1% TFA. The organic solvent was removed on a vacuum centrifuge and the remaining water was removed by lyophilization. The lyophilized peptides were dissolved in 10 mM phosphate buffer, pH 7.5 and the concentration was adjusted to 1 mM based on absorbance.

S.4 Peptide modification

General methods. The small molecule coupling partners (2-amino-*p*-cresol and 4-methylcatechol) were purchased from Sigma Aldrich and purified by sublimation.[2] The purified small molecules were stored frozen (at -80 °C) as a 500 mM solution in CH_3CN and diluted to 1 mM before use. Potassium ferricyanide (99.99% trace metal basis) was purchased from Sigma Aldrich and used without further purification. Commercial peptides were dissolved in ddH_2O to an approximate concentration of 1 mM. The dissolved peptides were stored between 4 °C and -20 °C to prevent degradation. When using high ionic strength buffers, peptide solutions were purified using Millipore ZipTip C18 Pipette Tips as specified by the supplier before analysis by MALDI-TOF MS.

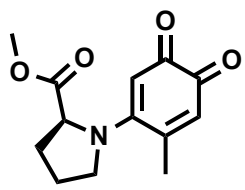
General method for the modification of commercial peptides. To a solution of peptide (1 nmol, final concentration 100 μM) in buffer was added 2-amino-*p*-cresol or 4-methylcatechol (1-5 nmol, from a 1 mM stock containing 0.2% CH_3CN, final concentration 100-500 μM). To these reagents was added 1 μL of a solution of $K_3Fe(CN)_6$ (10 or 50 mM stock solution, final concentration 1-5 mM) to start the reaction. The reaction was immediately vortexed and allowed to incubate at room temperature for the indicated time. The peptides were either purified or co-crystallized with matrix on the MALDI plate at the indicated time to stop the reaction. The percent modification was determined using MALDI-TOF MS. Given the high variability in signal in MALDI-TOF MS these values were used only for comparison.

Angiotensin modification for MS/MS analysis. To a solution of angiotensin (10 nmol, final concentration 100 μM) in 50 mM phosphate buffer, pH 7.5 was added 2-amino-*p*-cresol (10 nmol, final concentration 100 μM). $K_3Fe(CN)_6$ was added (10 μL of a 10 mM solution, final concentration 1 mM) to start the reaction. After 30 min, the reaction was purified on a C18 Sep-Pak (Waters, conditioned with CH_3OH, equilibrated and washed with H_2O with 0.1% TFA, and eluted with CH_3CN). The solvent was removed under reduced pressure and the peptide was redissolved in ddH_2O for analysis.

General method for the modification of synthetic peptides. To a solution of peptide (2 nmol, final concentration 100 μM) in buffer was added 2 equiv of 2-amino-*p*-cresol (4 nmol, final concentration 200 μM) followed by addition of 100 nmol of $K_3Fe(CN)_6$. The solution was immediately mixed after addition of the oxidant. Reactions were incubated at room temperature for the indicated time. The reactions were quenched by addition of buffered tris(2-carboxyethyl)phosphine hydrochloride (0.5 M solution of TCEP pH 7.0, 1 μmol) and analyzed by LC-MS.

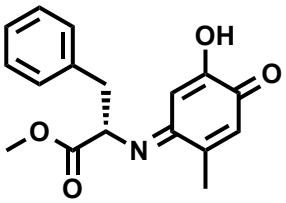
Method for the sequential modification of PAD(*p*AF)SWAG. To a solution of peptide (20 nmol, final concentration 100 μM) in phosphate buffer, pH 6.0 was added 2-amino-*p*-cresol (40 nmol, final concentration 200 μM) followed by $K_3Fe(CN)_6$ (1 μmol, 5 mM final concentration). The reaction was vortexed immediately following addition of the oxidant. After 30 min of incubation at room temperature the reaction was purified on a C18 Sep-Pak (conditioned with CH_3OH, equilibrated and washed with H_2O with 0.1% TFA, and eluted with CH_3CN). The solvent was removed under reduced pressure and the peptide was redissolved in 10 mM phosphate buffer, pH 7.5. The purified peptide was modified for a second time following the general method described above.

S.5 Small molecule synthesis.



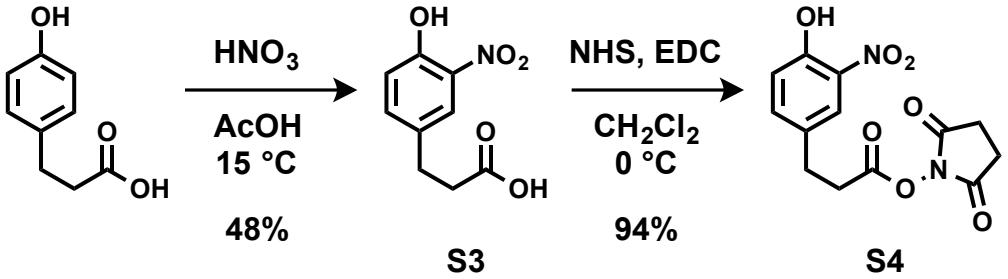
Synthesis of proline product (S1). To 150 mL of buffered ddH_2O (10 mM phosphate buffer, pH 7.5) was added 4-methylcatechol (24.2 mg, 0.2 mmol, purified by sublimation and recrystallization from toluene) in 400 μL of MeCN and L-proline methyl ester hydrochloride (33.1 mg, 0.2 mmol) in 1 mL 100 mM phosphate buffer pH 9.0. To the rapidly stirred solution was added potassium ferricyanide (690 mg, 2.1 mmol, as a solution in 50 mL of buffer). After stirring at rt for 20 min, the reaction mixture was extracted with 3 portions of 100 mL 30% 2-propanol in $CHCl_3$. The combined organic layers were dried over sodium sulfate and the solvent was removed *in vacuo*. The resulting deep purple solid was taken up in $CDCl_3$ for characterization ^1H NMR (600 MHz, $CDCl_3$): δ 6.21 (s, 1H), 5.49 (s, 1H), 4.55 (m, 1H), 3.80 (m, 1H), 3.77 (s, 3H), 3.71 (m, 1H) 2.43 (m, 1H), 2.33 (s, 3H), 2.04 (m, 1H), 2.12 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 24.06, 24.57,

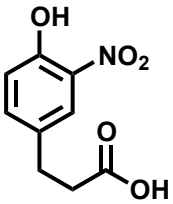
30.62, 53.11, 53.60, 63.81, 103.45, 132.12, 146.41, 157.62, 171.53, 176.37, 182.16. HRMS (ESI) calculated for $C_{13}H_{15}NO_4$ ($[M+H]^+$) 250.1074, found 250.1073.



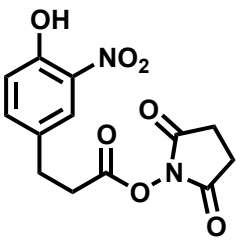
Synthesis of phenylalanine product (S2). To 90 mL of buffered ddH_2O (10 mM phosphate buffer, pH 7.5) was added 4-methylcatechol (12.4 mg, 0.1 mmol, purified by sublimation and recrystallization from toluene) in 200 µL of MeCN and L-phenylalanine methyl ester hydrochloride (21.6 mg, 0.1 mmol) in 1 mL of 100 mM phosphate buffer pH 9.0. To the rapidly stirred solution was added potassium ferricyanide (330 mg, 1 mmol, as a solution in 10 mL ddH_2O). After stirring at rt for 30 min, the solvent was removed *in vacuo* and the resulting solid was taken up in CD_3CN for characterization. ^{1}H NMR (600 MHz, CD_3CN): δ 7.31 (m, 2H, J = 8.2), 7.26 (m, 1H) 7.21 (m, 2H, J = 8.0), 6.21 (s, 1H), 5.37 (s, 1H), 4.55 (m, 1H), 3.73 (s, 3H), 3.32 (m, 1H), 3.19 (m, 1H), 2.09 (s, 3H). ^{13}C NMR (150 MHz, CD_3CN): δ 183.48, 171.74, 155.24, 146.88, 136.88, 130.73, 130.10, 129.81, 128.95, 98.07, 57.67, 53.43, 37.34, 18.17. HRMS (ESI) calculated for $C_{17}H_{18}NO_4$ ($[M+H]^+$) 300.1230, found 300.1230.

Scheme 1. Synthesis of *o*-nitrophenol NHS ester S4.





Synthesis of 3-(4-hydroxy-3-nitrophenyl) propionic acid (S3). The nitrophenol was synthesized as previously reported.[3] Briefly, to a solution of 3-(4-hydroxyphenyl)propionic acid (5 g, 30.1 mmol) in 25 mL acetic acid at 15 °C was added a solution of fuming nitric acid (1.6 mL, 33.8 mmol) in acetic acid (4 mL). The solution immediately turned orange. After 15 min the reaction was quenched by addition to ice water. The precipitate was filtered and dried. The yellow precipitate was recrystallized from 1:1 ethanol:water to afford 3.07 g of a yellow powder (48% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.47 (br s, 1H), 7.94 (d, 1H, J = 1.9), 7.45 (dd, 1H, J = 8.6, 1.9), 7.09 (d, 1H, J = 8.6), 2.94 (t, 2H, J = 7.4), 2.68 (t, 2H, J = 7.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.22, 153.86, 138.11, 133.47, 132.60, 124.22, 120.27, 35.19, 29.37. HRMS (ESI) calculated for $C_9H_8O_5N$ ($[M]^-$) 210.0408, found 210.0410 m/z.



Synthesis of 3-(4-hydroxy-3-nitrophenyl) propionic acid NHS ester (S4). The nitrophenol NHS ester was synthesized as previously reported.[3] To a solution of **S3** (1 g, 4.7 mmol) and *N*-hydroxysuccinimide (0.65 g, 5.7 mmol) in CH_2Cl_2 at 0 °C was added *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (1.09 g, 5.7 mmol). The reaction was stirred for 2 h and then diluted with DCM and washed with water. The combined organic layers were dried over sodium sulfate and the solvent was removed *in vacuo*. The reaction afforded 1.38 g of a yellow solid (94% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.49 (s, 1H), 7.98 (d, 1H, J = 2.1), 7.48 (dd, 1H, J = 8.6, 2.1), 7.12 (d, 1H, J = 8.6), 3.05 (t, 2H, J = 7.3), 2.93 (t, 2H, J = 7.3), 2.83 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.06, 167.56, 154.09, 138.00, 133.54, 131.40, 124.52, 120.49, 32.48, 29.43, 25.70. HRMS (ESI) calculated for $C_{13}H_{12}O_7N_2$ ($[M-H]^-$) 307.0572, found 307.0568 m/z.

S.6 UV-Vis kinetics

Pseudo-first order reactions. To 50 mM phosphate buffer (either pH 6.0 or 7.5) was added 10 µL of a 10 mM solution of 4-methylcatechol (final concentration 100 µM) and 100 µL of a 10 mM solution of the coupling partner (*p*-toluidine, H-Pro-OMe, or H-Phe-OMe, final concentration 1 mM) for a volume of 900 µL. This solution was used as a blank before measurement. The absorbance at 520 nm was monitored every 1 s for 3600 s using a Cary 50 spectrophotometer. Within the first 1 s of measurement, 100 µL of a 100 mM solution of $K_3Fe(CN)_6$ (final concentration 10 mM) was added for a final reaction volume of 1 mL. A control reaction was run that excluded the amine coupling partner to verify the increase in absorbance at 520 nm did not arise from simple oxidation of the catechol substrate. The data were normalized to account for differences in extinction coefficients of the products. The raw data are shown in Supporting Information Figure S12.

Second order reactions. To 50 mM phosphate buffer pH 7.5 was added 10 μL of a 10 mM solution of 4-methylcatechol (final concentration 100 μM) and 10 μL of a 10 mM solution of H-Pro-OMe (final concentration 100 μM) for a final volume of 900 μL. This solution was used as a blank before measurement. The absorbance at 520 nm was monitored every 0.1 s for 3600 s at 25 °C using a Cary 50 spectrophotometer outfitted with a temperature control unit. Within the first 5 s of measurement, 100 μL of a 100 mM solution of $K_3Fe(CN)_6$ (final concentration 10 mM) was added. The reactions were performed in triplicate. A calibration curve was constructed by measuring the absorbance of reactions set up under pseudo-first order conditions with 10, 25, 50, 100, 250, 500 and 1000 μM catechol. The second order rate constants were calculated using the Cary software.

S.7 Aminophenol substrate synthesis

Synthesis of *o*-aminophenol rhodamine. The fluorescent aminophenol was synthesized as previously reported.[3] To a solution of Rhodamine B piperazine amide[4] (50 mg, 0.1 mmol) in DMF (1 mL) was added diisopropylethylamine (35 μL, 0.2 mmol) followed by **S4** (30 mg, 0.1 mmol). After stirring for 2 h the solvent was removed *in vacuo*. The product was purified on silica gel with a gradient of CH_2Cl_2:CH_3OH (0 to 10% CH_3OH). HRMS (ESI) calculated for $C_{41}H_{45}O_6N_5$ ($[M+H]^+$) 704.3433, found 704.3436 m/z. To a 1 mg portion of the nitrophenol rhodamine in 100 μL of 100 mM phosphate buffer, pH 6.5 was added an equal volume of a freshly prepared 100 mM solution of sodium dithionite in 100 mM phosphate buffer, pH 6.5. After 20 min, the excess dithionite was removed by purification on a C18 Sep-Pak according to the manufacturer's instructions. The eluent was concentrated to dryness. The rhodamine aminophenol was resuspended in DMF and stored frozen as a 50 mM solution.

Synthesis of *o*-aminophenol 5k-PEG. The *o*-aminophenol PEG substrate was synthesized following a previously published protocol.[3] Briefly, to a solution of mPEG-NH_2 (MW=5000, 115 mg, 0.023 mmol) in CH_2Cl_2 (1 mL) was added triethylamine (7 μL, 0.05 mmol) and **S4** (99 mg, 0.32 mmol as a 0.4 M solution in DMSO). The solution was stirred for 2 h at rt and then the solvent was removed *in vacuo*. Excess **S4** was precipitated by the addition of water. The resulting solution was filtered through a 0.22 micron filter and three Sephadex size exclusion columns (one NAP-10 and two NAP-25, GE Healthcare) according to the manufacturer's instructions. The modified PEG was further purified by precipitation from CH_2Cl_2 with Et_2O three times. Reduction of the nitrophenol was accomplished with sodium dithionite. To a 1 mM solution of the modified PEG was added an equal volume of a freshly prepared 120 mM solution of sodium dithionite in 100 mM phosphate buffer, pH 6.5. Excess dithionite was removed by purification with three Sephadex size exclusion columns. The purified aminophenol PEG was lyophilized and resuspended in 10 mM phosphate buffer, pH 7.2. The concentration was adjusted to 1 mM by measuring the aminophenol absorbance at 290 nm. The final solution of *o*-aminophenol PEG was stored in single use aliquots at -20 °C until use.

S.8 Protein expression and purification

The QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce N-terminal proline mutations to GFP and TMV.

The N-terminus of a previously generated GFP mutant was changed from alanine to proline using the primers :

sense : 5' - GATATACATATGCCCAAAACGGGCGAGGAGCTGTCCACC - 3'
antisense : 5' - GGTGAACAGCTCCTCGCCCGTTTTGGGCATATGTATATC - 3'

The N-terminus of TMV mutants was extended from the native N-terminus of SYS to PAGSYS using the primers :

sense : 5' - GAAGGAGATATACATATGCCTGCCGGCAGCTATAGCATTACC -3'
antisense : 5' - TGCTATAGCTGCCGGCAGGCATATGTATATCTCCTTCTTAAG - 3'

The TMV coat protein already had the following mutations : K53R, K68R (RR-TMV) and either S123C or T104K.

Expression and purification of TMV. The plasmids were transformed into BL21 DE3 RIL Codon+ cells for expression. The cells were grown in 1 L of LB with 100 μg/L of ampicillin at 37 °C until induction, at which point the temperature was lowered to 30 °C. The cells were induced by addition of 100 μL of 0.3 M isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of ca. 0.6. The cells were collected by centrifugation at 7000 rpm for 20 min. The cells were resuspended in lysis buffer (20 mM TEA, pH 7.2) supplemented with 0.2 mg DNase and RNase A and 25 mg of $MgCl_2$. The cells were lysed by sonication and the cell debris was removed by centrifugation at 14000 rpm for 45 min. The clarified lysate was treated with

an equal volume of 100% $(NH_4)_2SO_4$ and rotated at 4 °C for 10 min to precipitate the protein. The precipitated protein was collected by centrifugation at 16000 g for 45 min and then resuspended in 10 mL lysis buffer. The resuspended protein was applied to a DEAE-Sephadex column and purified using the following gradient (buffer A: 20 mM TEA, pH 7.2, buffer B: 20 mM TEA, pH 7.2 with 1 M NaCl): 0 min (0% B), 60 min (30% B), 61 min (100% B), 90 min (100% B), 91 min (0% B), 120 min (0 %B) with a flow rate of 3 mL/min. The fractions containing pure TMV were combined and then buffer exchanged into 10 mM phosphate buffer, pH 7.5 using spin concentrators with a 100 kDa MWCO.

Expression and purification of wt-GFP and Pro-GFP. The plasmids were transformed into T7 Express *lysY/I^q* cells for expression. The cells were grown in 1 L of LB with 100 μg/L of ampicillin at 37 °C until induction, at which point the temperature was lowered to 16-25 °C. The cells were induced by addition of 1 mL of 0.3 M IPTG. The cells were collected by centrifugation at 8000 rpm for 15 min and then resuspended in 10-15 mL lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.0). The cells were lysed by sonication. The lysate was clarified by centrifugation at 9000 rpm for 1 h. The lysate was applied to 5-7 mL of chitin resin pre-rinsed with 100 mL of lysis buffer. The resin was rinsed with 100 mL wash buffer (20 mM Tris, 500 mM NaCl 1 mM EDTA, pH 8.0) and then incubated with 20 mL cleavage buffer (wash buffer with 50 mM sodium 2-mercaptoethanesulfonate (MESNa)) for 48 h. The cleaved protein was eluted with an additional 20 mL wash buffer and then buffer exchanged into 10 mM phosphate buffer, pH 7.5 using spin concentrators with a 10 kDa molecular weight cutoff (MWCO).

S.9 Protein Modification

Disulfide exchange with Ellman's reagent. Free cysteines were protected from potential modification by disulfide formation with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB). To a solution of PAG RR-TMV S123C (780 μL of a 100 μM solution) was added DTNB (20 μL of a 20 mM solution in 100 mM phosphate buffer, pH 7.2 with 1 mM EDTA). The reaction mixture was incubated at rt for 15 min and then the excess DTNB was removed by repeated (3-6 times) centrifugal filtration against a 100 kDa MWCO membrane. To reduce the disulfide, approximately 25 equiv of TCEP (as a 0.5 M solution, pH 7.0) was added to the protein sample.

General procedure for oxidative coupling on proteins. To a solution of proline terminal protein (PAG RR-TMV, Pro-GFP, creatine kinase, 5-20 μM) in 10 mM phosphate buffer, pH 7.5 was added 2-10 equiv of the *o*-aminophenol coupling partner (20-100 μM). The solution was briefly vortexed and then 10-20 equiv (relative to the *o*-aminophenol) of $K_3Fe(CN)_6$ (as a 50 mM solution in 10 mM phosphate buffer, pH 7.5) was added. After 20-30 min, the reaction was purified using a Sephadex size exclusion column (GE Healthcare) according to the manufacturer's instructions or using a 0.5 mL centrifugal filter with an appropriate molecular weight cut off (MWCO, Millipore). Modification was monitored by SDS-PAGE or LC-MS.

Oxidative coupling and tryptic digestion. To a solution of proline-GFP (20 μM) in 10 mM phosphate buffer, pH 7.5 was added 5 equiv of 2-amino-*p*-cresol (100 μM). The solution was briefly vortexed and then 10 equiv of $K_3Fe(CN)_6$ (1 mM as a solution in 10 mM phosphate buffer, pH 7.5) was added. After 30 min, the reaction was purified using a 0.5 mL centrifugal filter with a 10 kDa MWCO. For the tryptic digest, a modified procedure from Strader *et. al*. was followed.[5] To a 40 μL solution of the modified protein (20 μg) in 50 mM Tris buffer, pH 7.6 was added 160 μL MeCN and 2 μL of a 100 mM solution of $CaCl_2$. To the denatured protein solution was added 400 ng trypsin. After incubation at 37 °C for 1 h, 8.4 μL of a freshly made 500 mM DTT solution was added. The resulting solution was incubated at 37 °C for 1 h. The reaction solvent was removed from the digested protein sample by vacuum centrifugation. After reaching dryness, the resulting solid was resuspended in ddH_2O and lyophilized. The digested protein was resuspended in ddH_2O and analyzed by LC-MS/MS.

Cysteine alkylation with fluorescent maleimides. A solution of PAG RR-TMV S123C (100 μM) in 10 mM phosphate buffer, pH 8.0 was incubated with 1 equivalent of an Alexa Fluor maleimide (Alexa Fluor 488 C_5-maleimide, 20 mM in DMSO) for 2 h. Unreacted dye was removed with a NAP-10 Sephadex size exclusion column (GE Healthcare) and by repeated centrifugal filtration against a 100 kDa MWCO membrane.

Modification with NHS PEG. A solution of creatine kinase (100 μM) in 10 mM phosphate buffer, pH 7.5 was incubated with NHS-functionalized PEG (tBoc-PEG-succinimidyl carboxymethyl, MW = 5000, Laysan Bio., Inc) for 2 h at room temperature. The PEG was dissolved in ddH_2O immediately prior to use. The reaction was analyzed by SDS-PAGE without any prior purification.

S.10 References

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S.11 Aminophenol reactivity with native amino acids on peptides

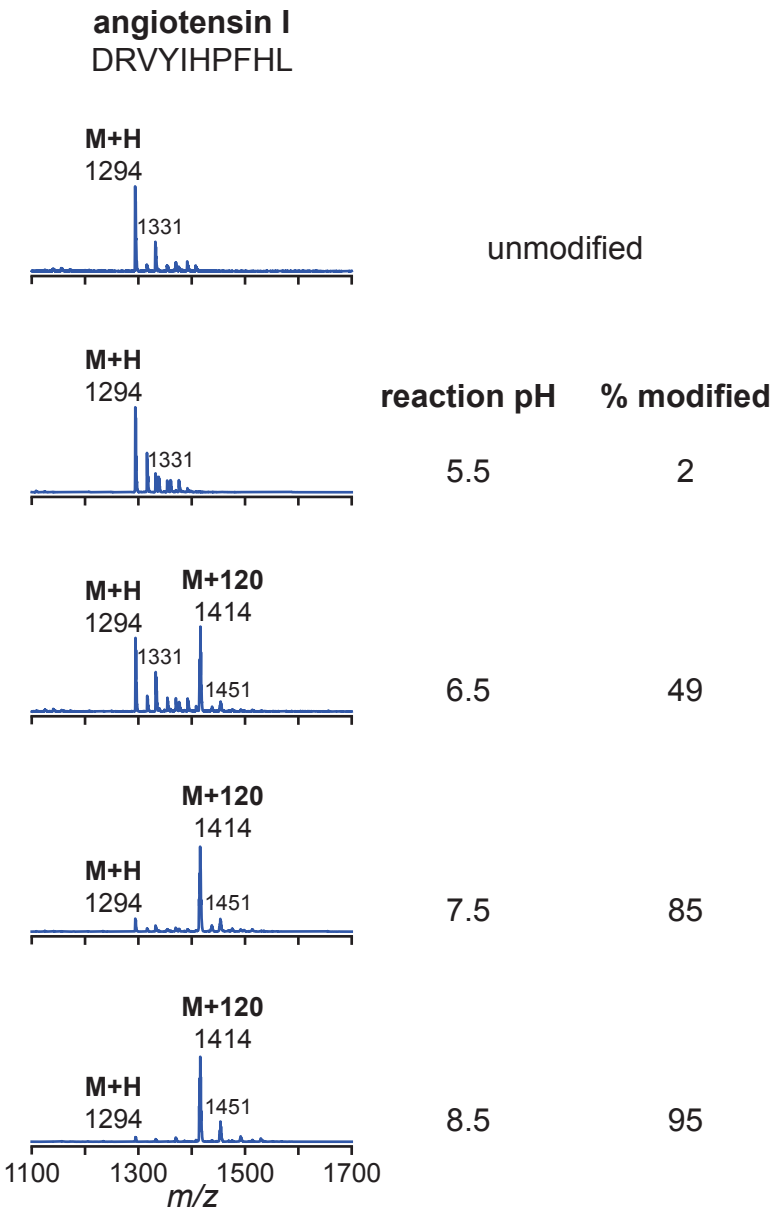


Figure S1. The effect of pH on the reactivity of the N-terminus with *o*-aminophenols was tested. Angiotensin I (100 µM) was reacted with 2-amino-*p*-cresol (100 µM) in the presence of $K_3Fe(CN)_6$ (1 mM) in 10 mM phosphate buffer at a pH range of 5.5-8.5 (expected mass addition = +120). For clarity, the peaks for the potassium adducts are also labeled.

S.12 MS/MS of modified angiotensin

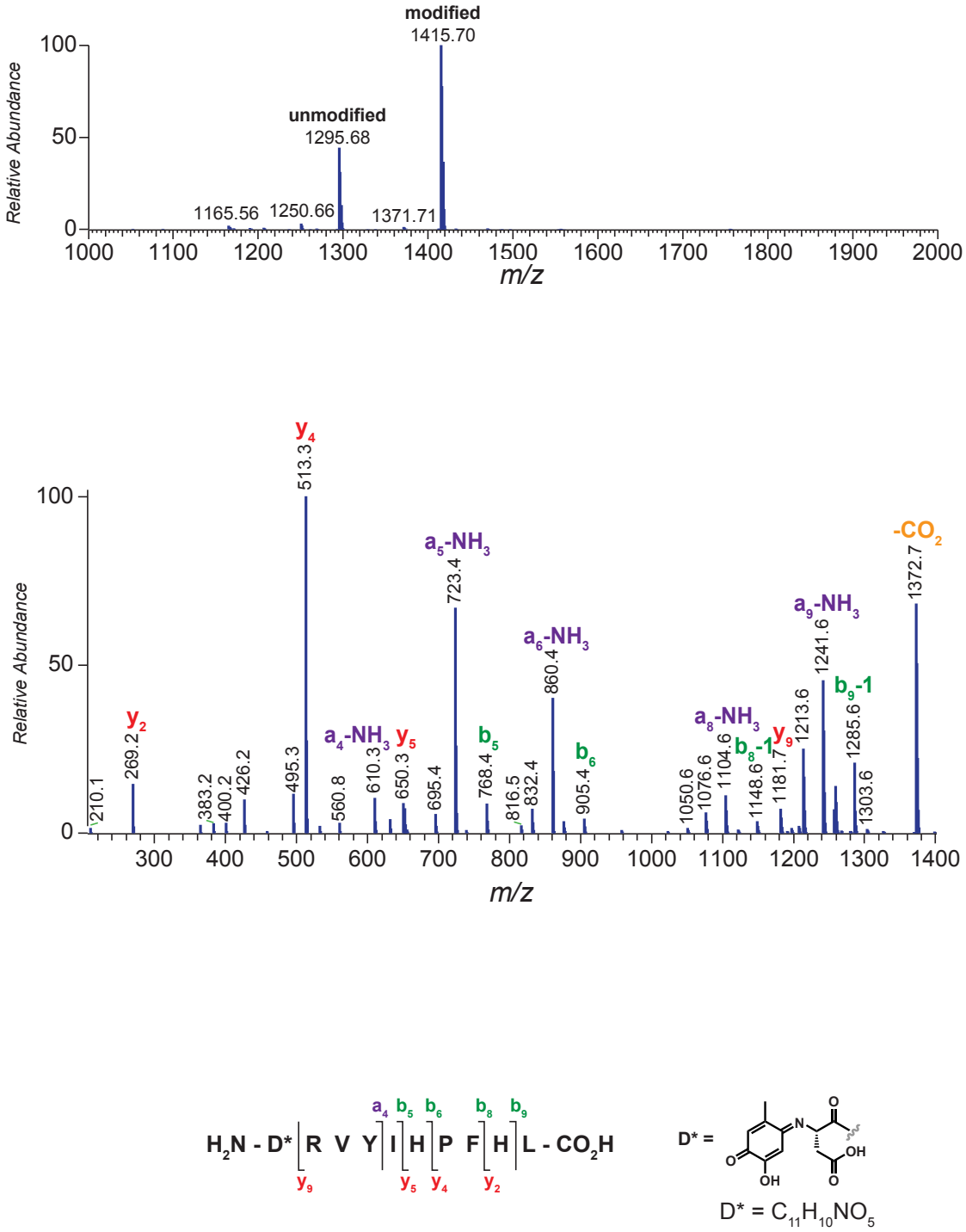


Figure S2. MS/MS analysis of modified angiotensin I. The *y* ions are shown in red, the *b* ions are shown in green, and the *a* ions (with neutral losses of ammonia) are shown in purple. The analysis is consistent with modification at the N-terminus.

S.13 Oxidative coupling on peptide substrates

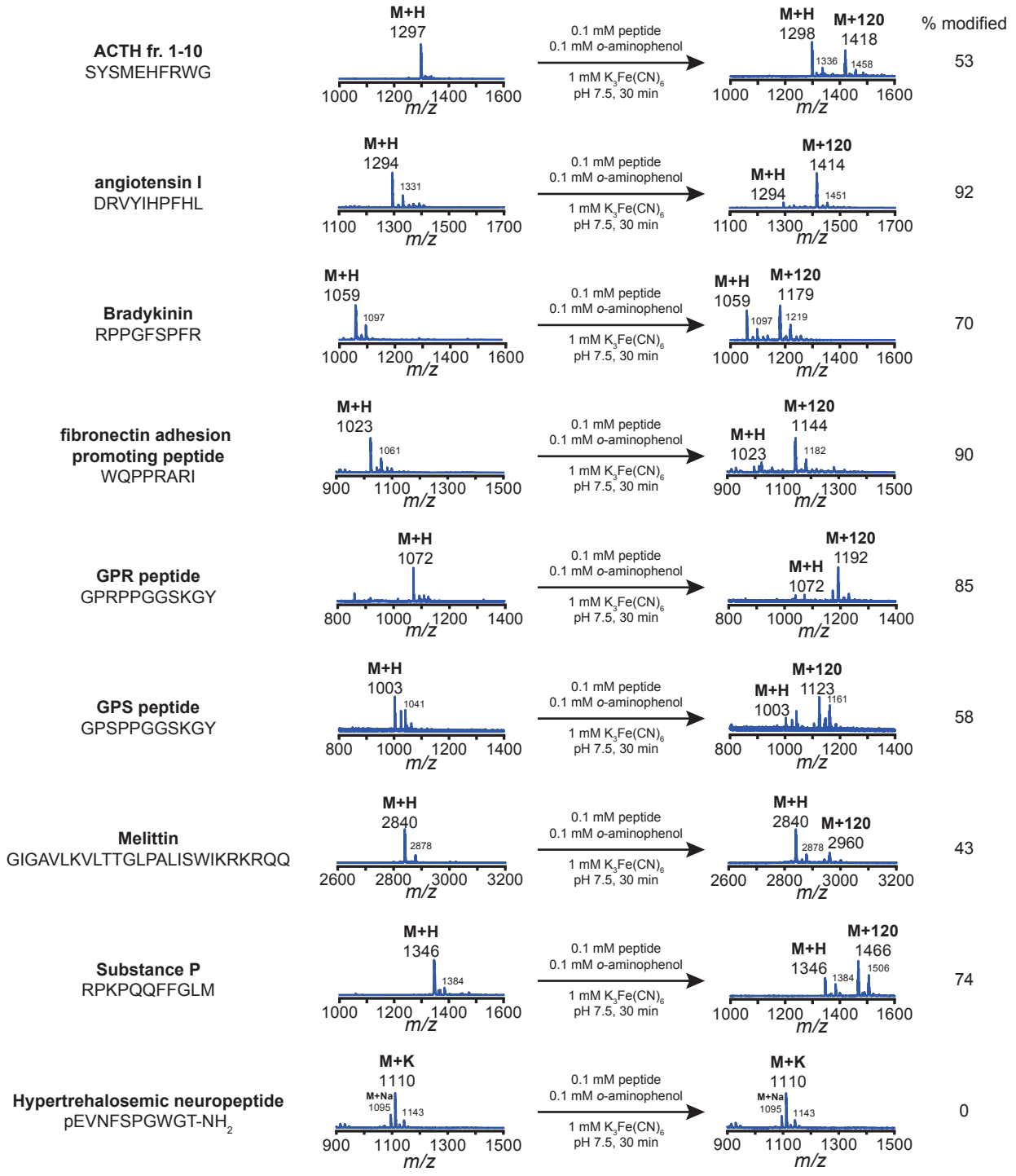


Figure S3. Several peptides were screened for their ability to react with *o*-aminophenols in the presence of ferricyanide. Reactions were run with approximately 0.1 mM peptide, 0.1 mM *o*-aminophenol and 1 mM ferricyanide in phosphate buffer, pH 7.5. All of the peptides showed some degree of modification (expected mass addition = +120), with the exception of the hypertrehalosemic neuropeptide. For clarity, the peaks for the potassium adducts are also labeled.

S.14 Optimization of reactivity on peptide substrates

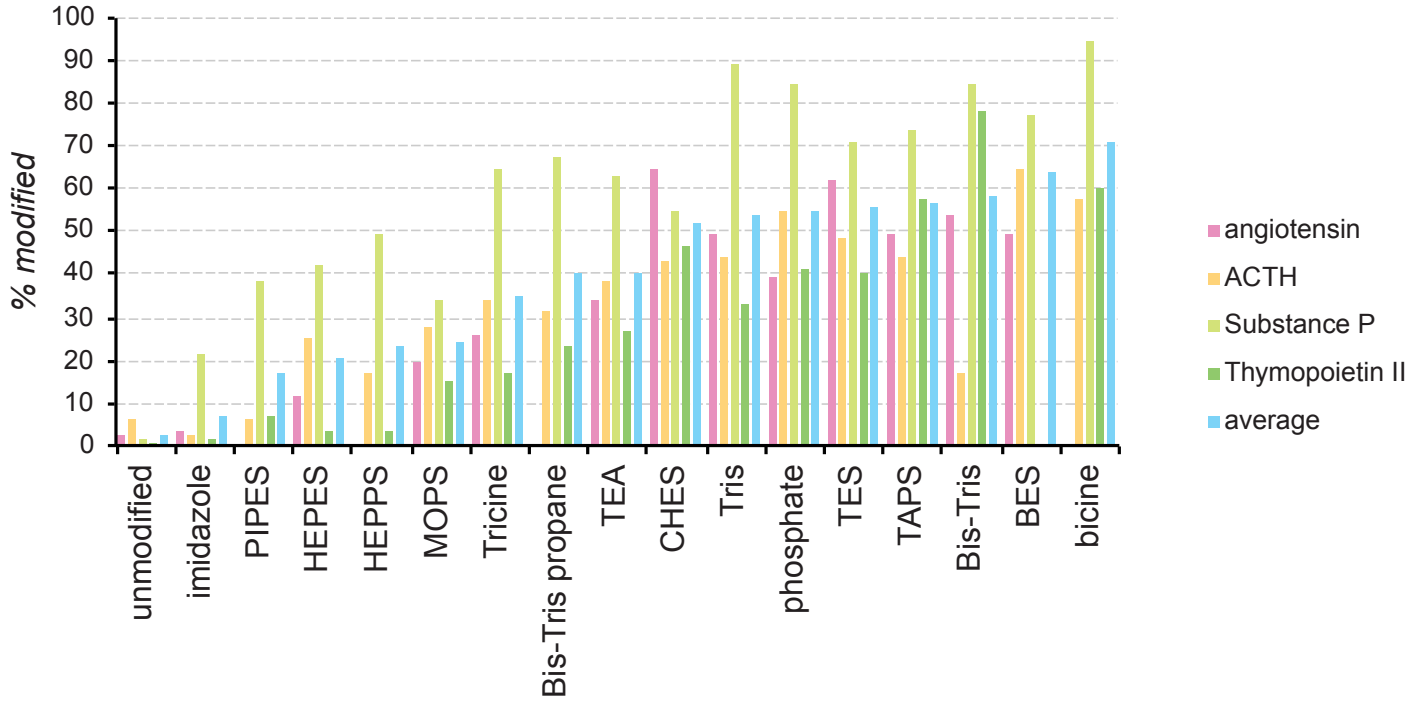


Figure S4. Peptides were screened in several different buffers at pH 7.5 to determine the buffer salt compatibility of the reaction. Most buffers showed no effect on the reaction; however a few buffers inhibited the reaction. The buffers that diminished reactivity generally contained a morpholino or piperazine moiety. Reactions were run with approximately 0.1 mM peptide, 0.1 mM *o*-aminophenol, and 1 mM ferricyanide at pH 7.5 for 30 minutes.

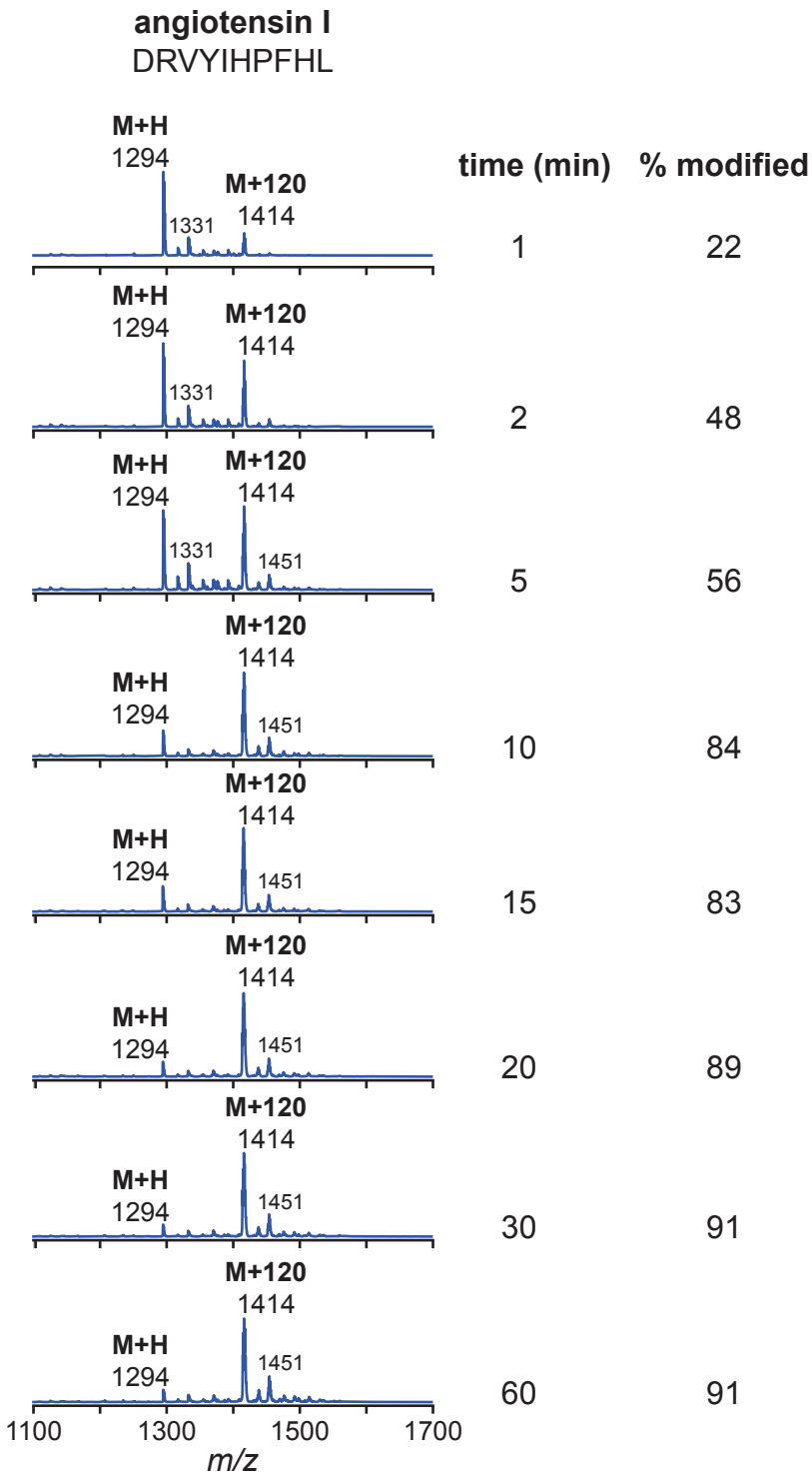


Figure S5. The time course of the reaction was assayed using angiotensin I as a substrate. The reactions were co-crystallized with matrix on a MALDI plate at the indicated time (expected mass addition = +120). For clarity, the peaks for the potassium adducts are also labeled.

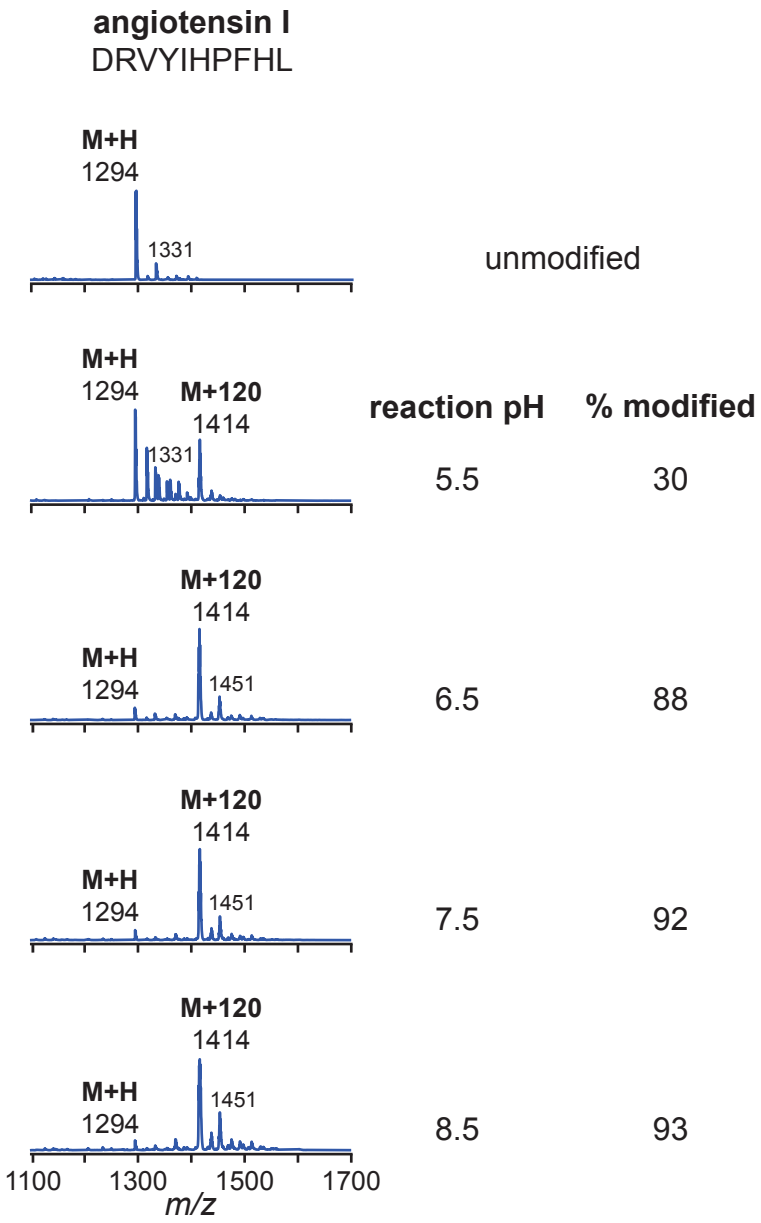


Figure S6. The effect of pH on the reactivity of the N-terminus with *o*-catechols was tested. Angiotensin I (100 µM) was reacted with 4-methylcatechol (100 µM) in the presence of $K_3Fe(CN)_6$ (1 mM) in 10 mM phosphate buffer at a pH range of 5.5-8.5 (expected mass addition = +120). For clarity, the peaks for the potassium adducts are also labeled.

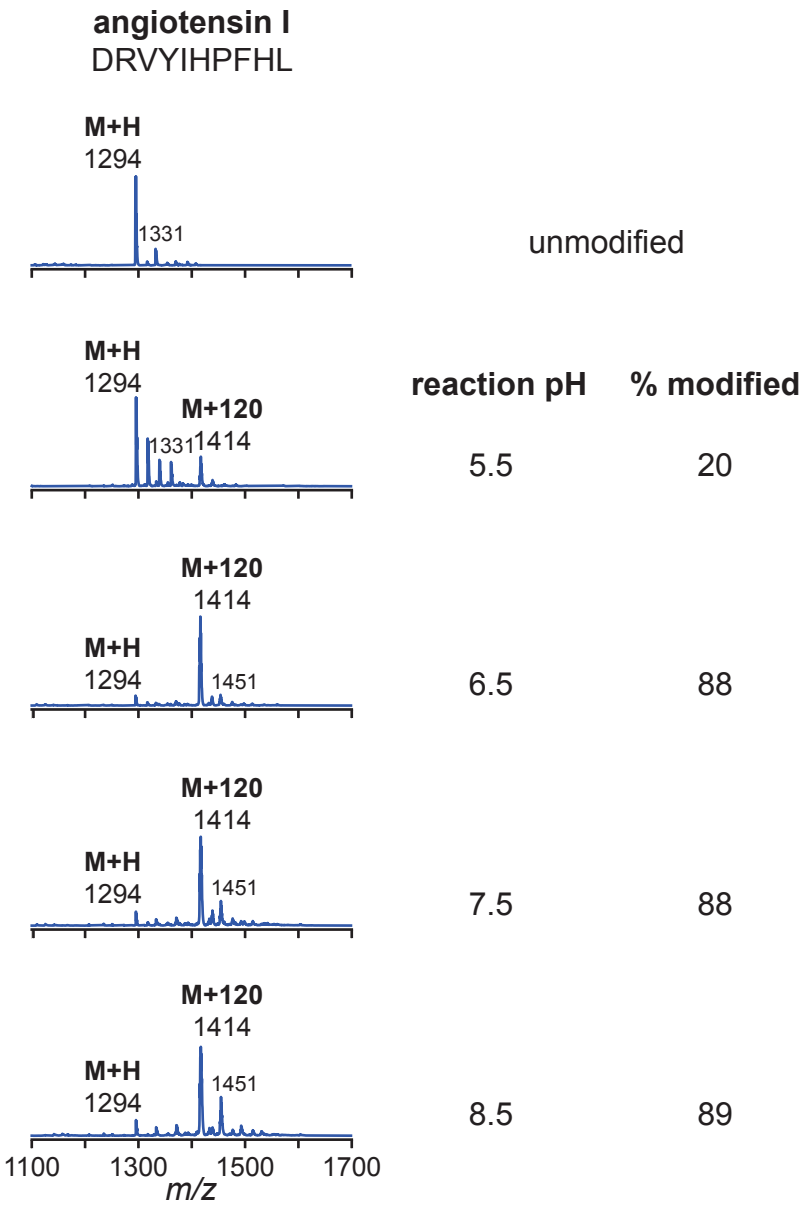


Figure S7. The effect of pH on the reactivity of the N-terminus with *o*-aminophenols was tested. Angiotensin I (100 µM) was reacted with 2-amino-*p*-cresol (100 µM) in the presence of $NaIO_4$ (1 mM) in 10 mM phosphate buffer at a pH range of 5.5-8.5 (expected mass addition = +120). For clarity, the peaks for the potassium adducts are also labeled.

S.15 MS of N-terminal variant peptides (XADSWAG) after modification

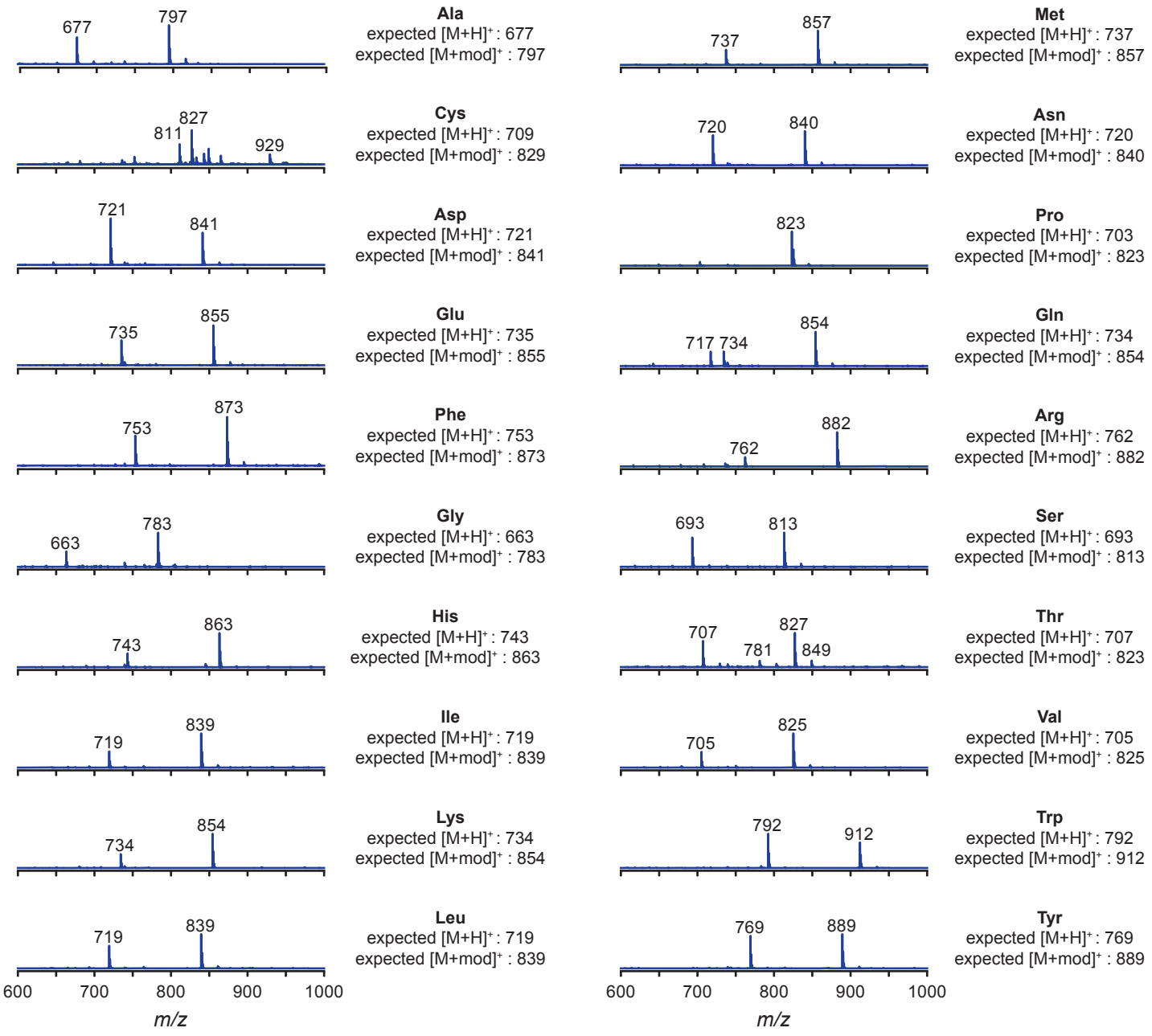


Figure S8. Representative mass spectra of XADSWAG peptides after reaction with 2 equiv of 2-amino-*p*-cresol in the presence of 5 mM ferricyanide.

S.16 Characterization of small molecule products

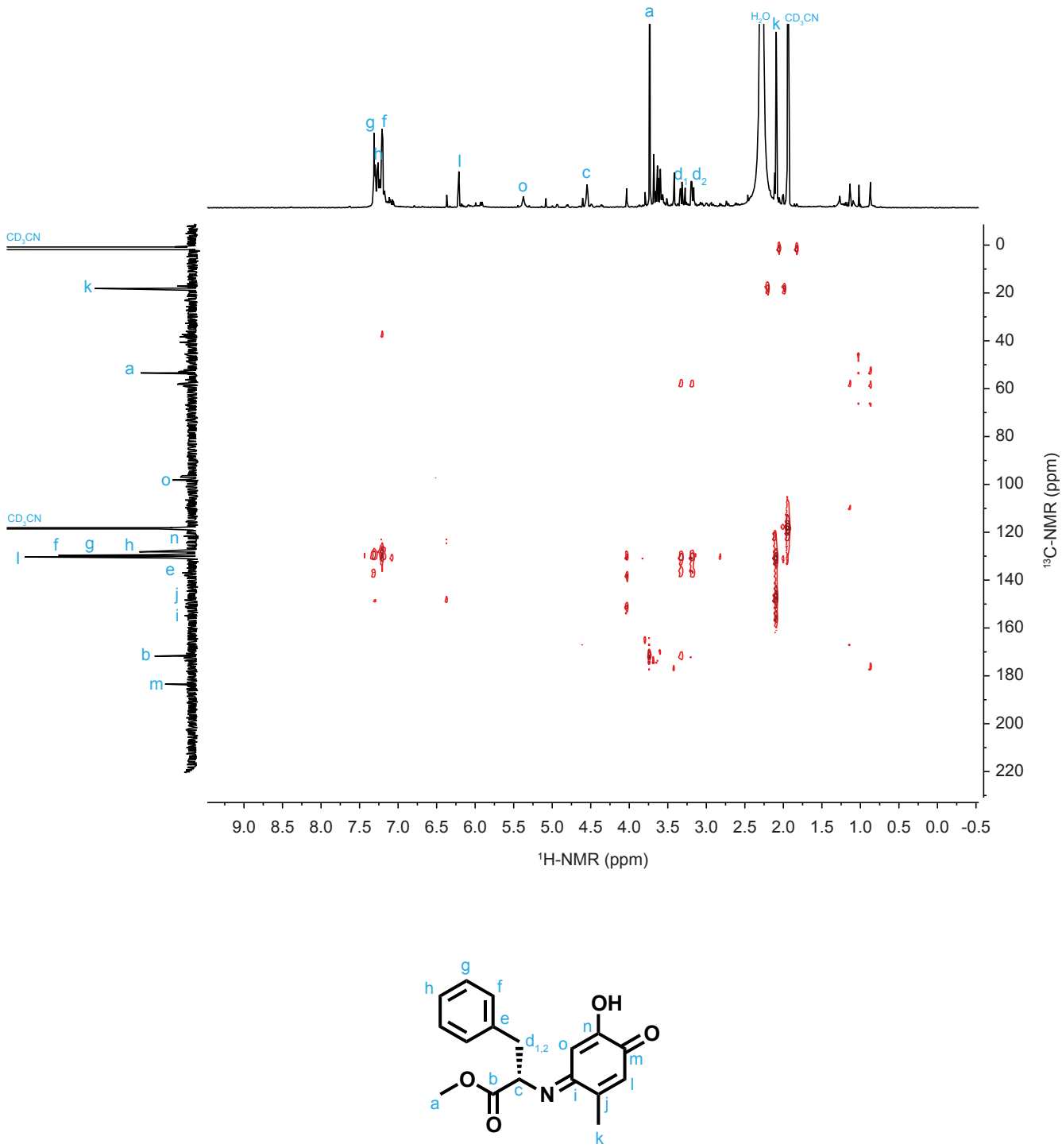


Figure S9. HMBC spectrum of **S2** in CD_3CN.

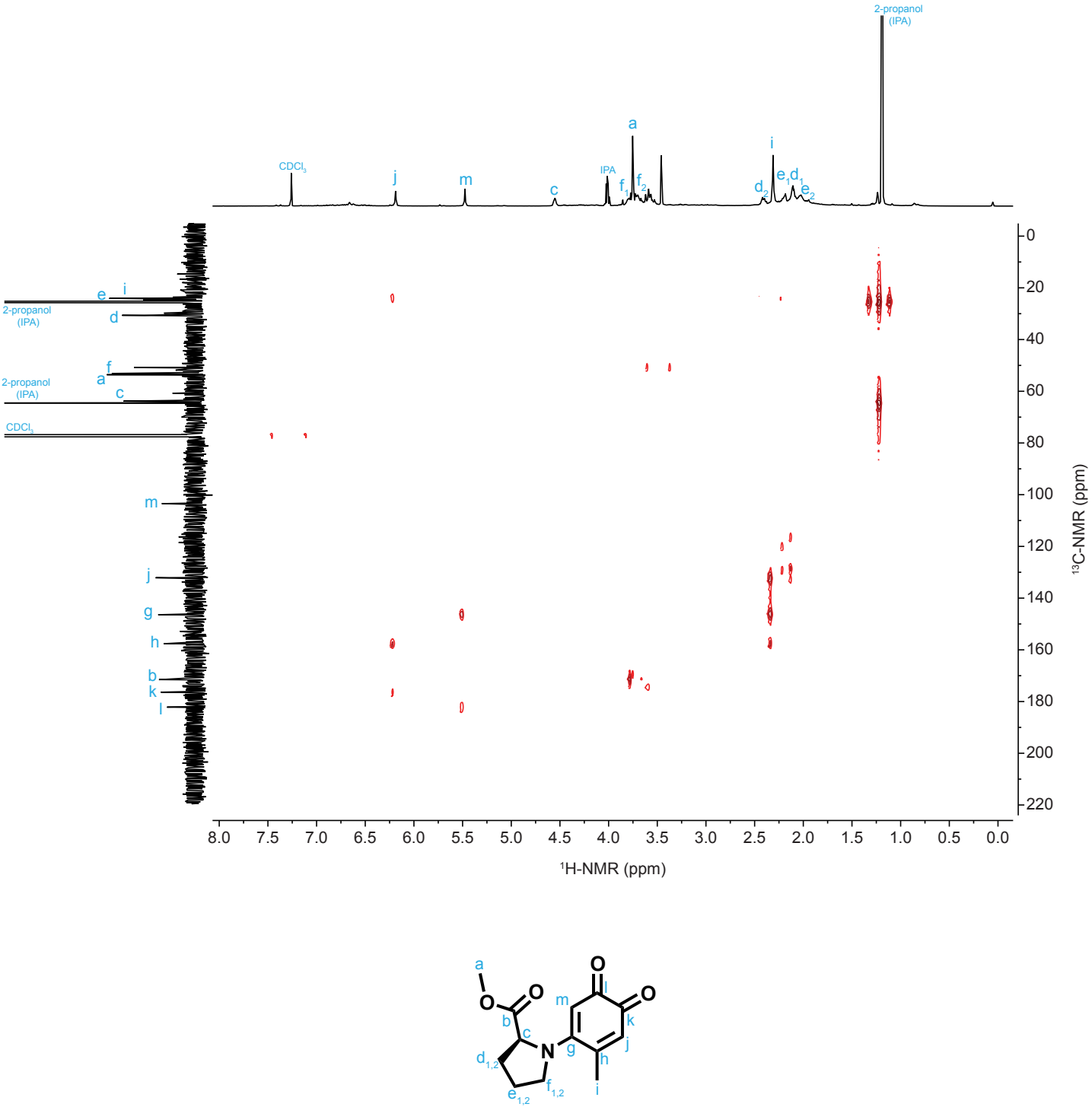


Figure S10. HMBC spectrum of **S1** in $CDCl_3$.

S.17 Stability of proline product

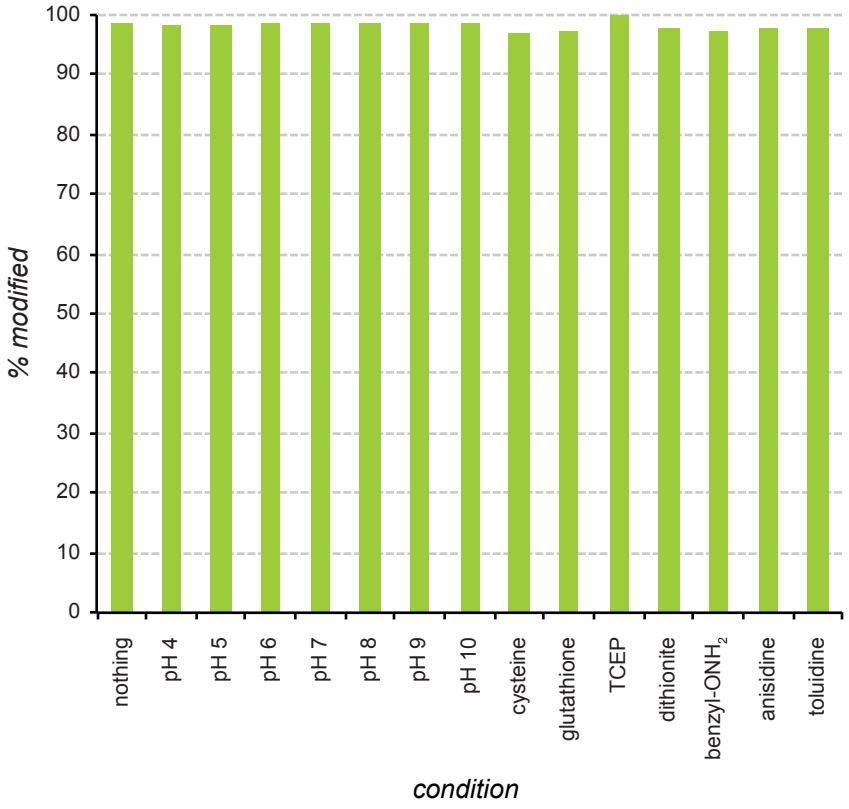
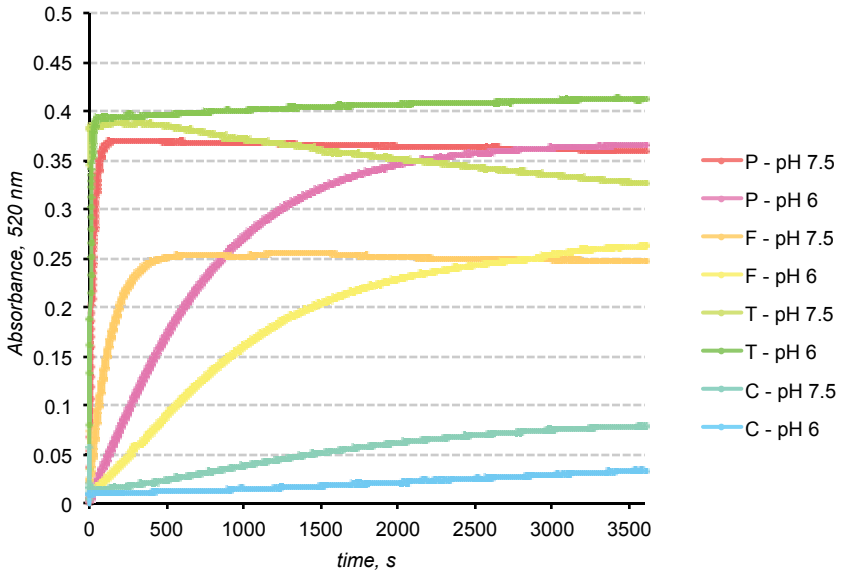


Figure S11. The stability of the proline product was assayed by LC-MS. Modified PADSWAG (100 μM) was treated with 10 mM buffer or additive for 8-18 h and then the level of modification was determined by LC-MS.

S.18 UV-Vis kinetics of oxidative coupling reactions

a



b

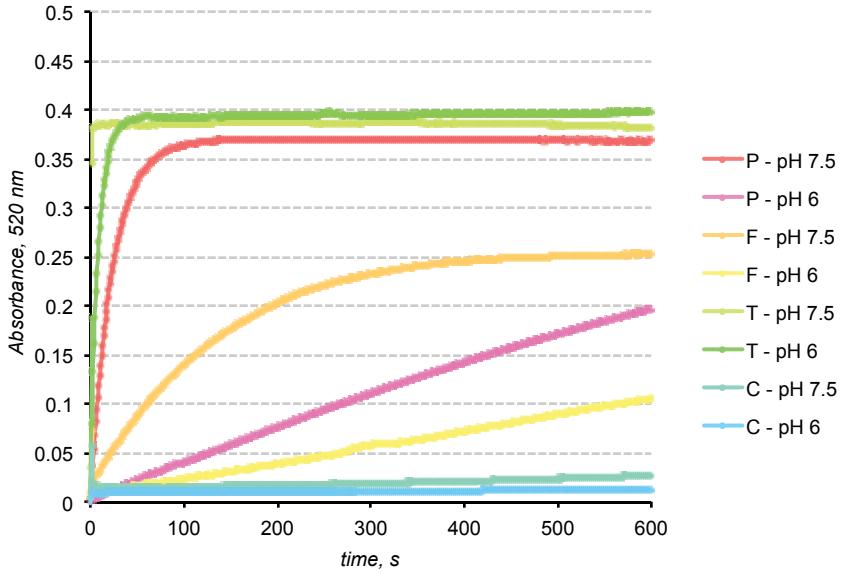


Figure S12. UV-Vis kinetics of small molecule coupling reactions. Reactions were run under pseudo-first order conditions. 100 μM 4-methylcatechol was reacted with 10 equiv of different amine coupling partners (P = H-Pro-OMe, F = H-Phe-OMe, T = toluidine, C = no amine) and 100 equiv $K_3Fe(CN)_6$ at either pH 6.0 or 7.5. Non-normalized data is shown for (a) 1 h or (b) 10 min.

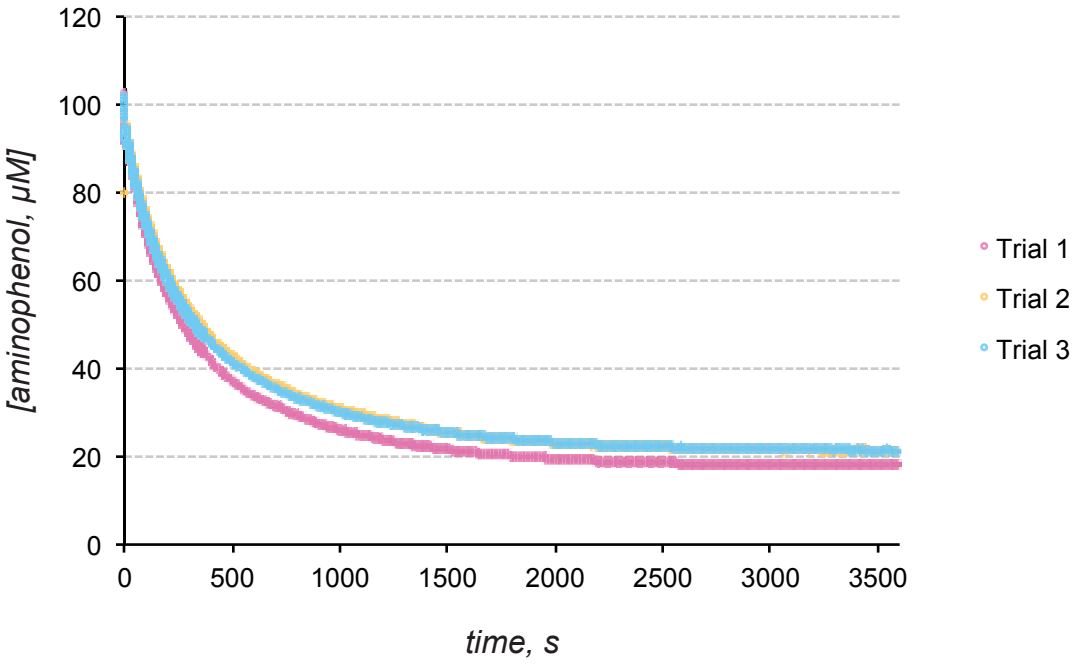


Figure S13. Plot of calculated aminophenol concentration vs. time used to determine the second order rate constant.

S.19 Preferential modification of pAF residues over N-termini

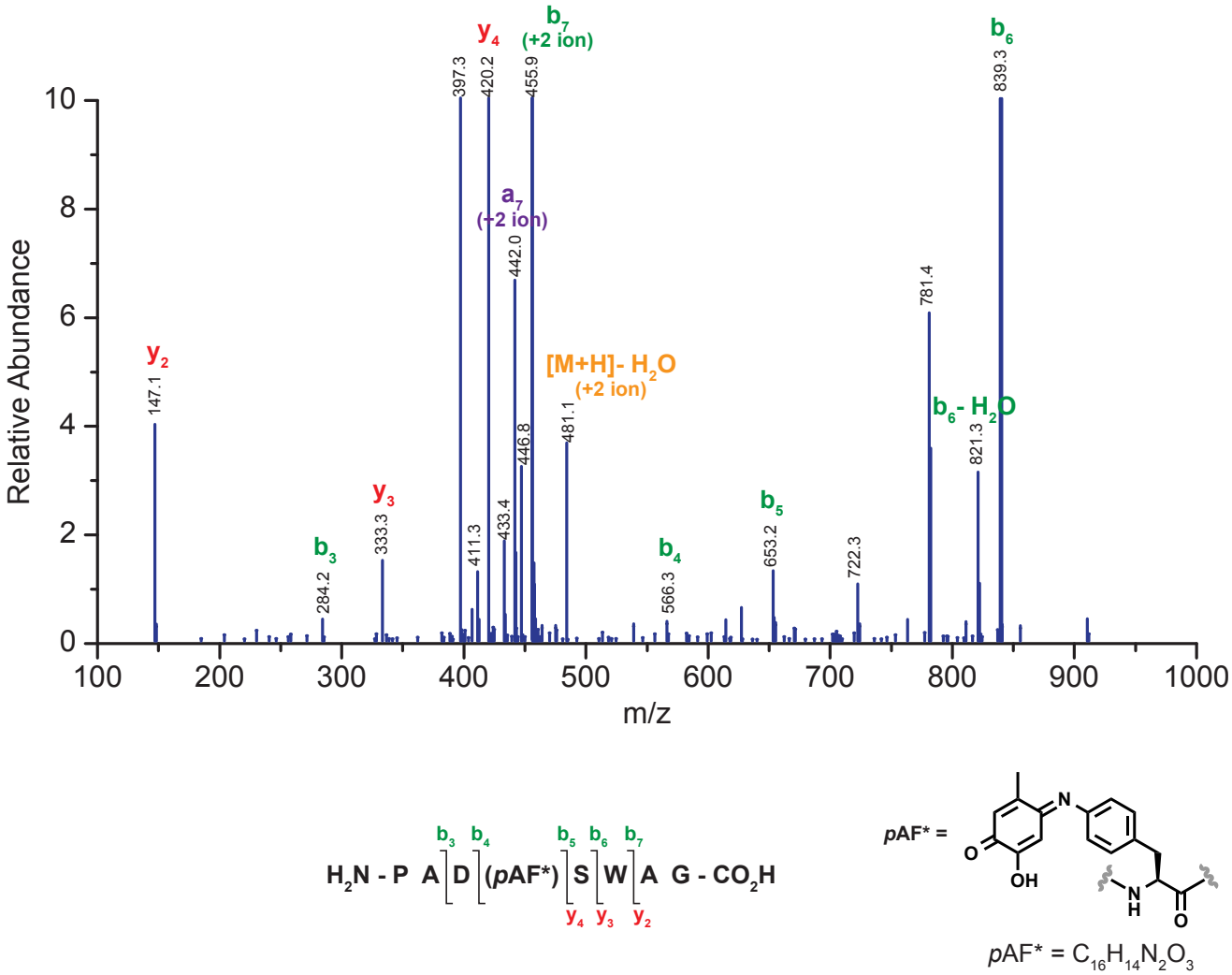
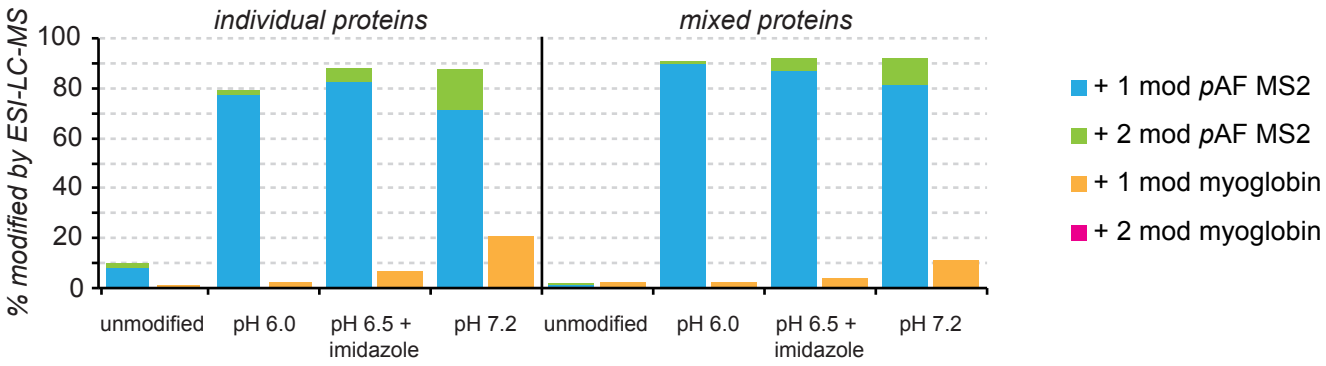


Figure S14. MS/MS analysis of PAD(*p*AF)SWAG modified at pH 6.0. The *y* ions are shown in red, the *b* ions are shown in green (with neutral losses of water), and the *a* ions are shown in purple. The analysis is consistent with modification at the *p*-aminophenylalanine residue.

a



b

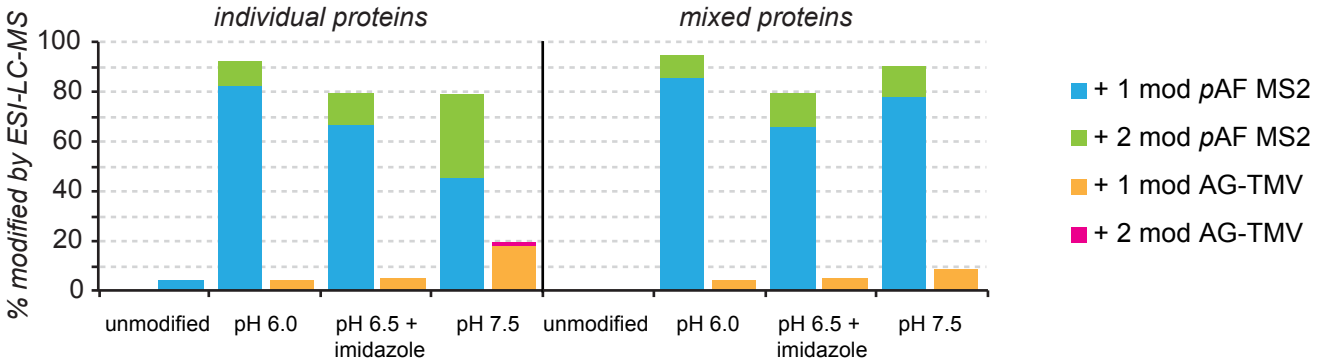
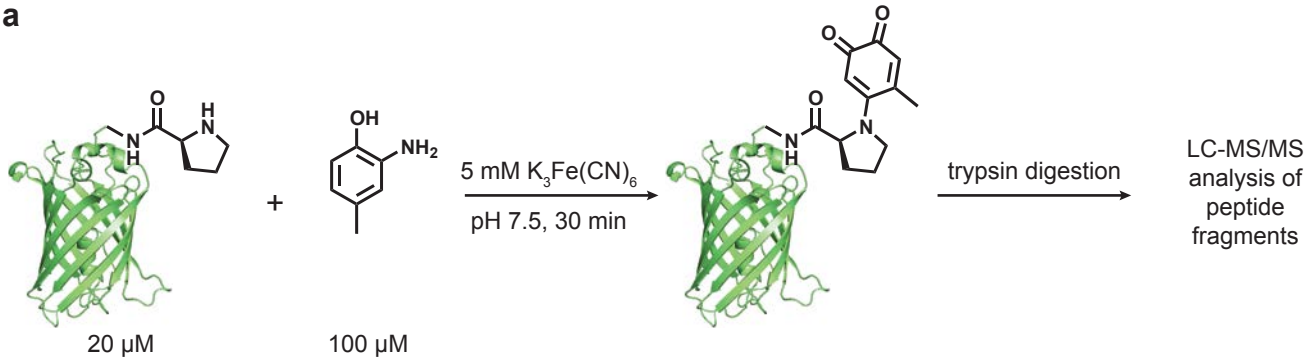


Figure S15. The relative reactivity of *p*AF residues and N-terminal residues was evaluated under several conditions. Proteins (20 μM) were reacted with 4 equiv of 2-amino-*p*-cresol (80 μM) with 1 mM ferricyanide in 10 mM buffer at the indicated pH for 30 min (reactions with imidazole contained 10 mM of the additive).

S.20 LC-MS/MS analysis of modified proline-GFP

a



b

P K T G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R **Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I M A D K Q K N G I K V N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q** S A **L S K D P N E K R D H M V L L E F V T A A G I T L G M D E L Y K**

c

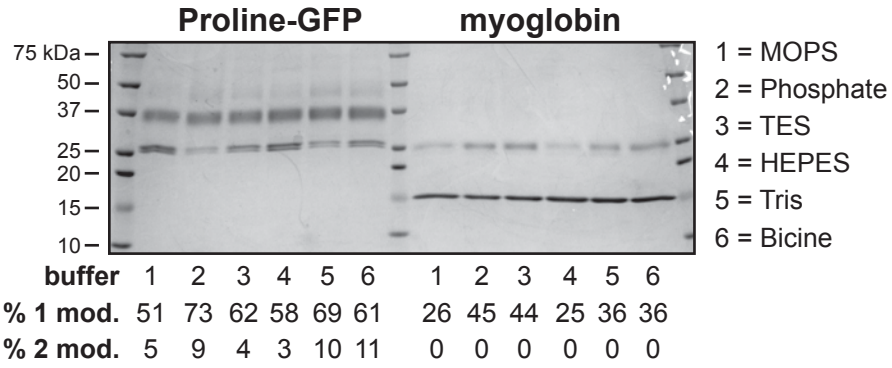
start	end	sequence
1	8	**(P+AP)**KTGEELF
1	10	**(P+AP)**KTGEELFTG
1	15	**(P+AP)**KTGEELFTGVVPIL
1	17	**(P+AP)**KTGEELFTGVVPILVE
1	26	**(P+AP)**KTGEELFTGVVPILVELDGDVNGHK
2	26	KTGEELFTGVVPILVELDGDVNGHK
3	45	TGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLK
46	52	FICTTGK
74	85	YPDHMKQHDFFK
80	96	QHDFFKSAMPEGYVQER
86	107	SAMPEGYVQERTIFFKDDGNYK
96	109	RTIFFKDDGNYKTR
97	111	TIFFKDDGNYKTRAE
108	122	TRAEVKFEGDTLVNR
110	126	AEVKFEGDTLVNRIELK
125	142	LKGIDFKEDGNILGHKLE
127	146	GIDFKEDGNILGHKLEYNYN
141	158	LEYNYNSHNVYIMADKQK
159	168	NGIKVNFKIR
167	204	IRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQ
207	221	LSKDPNEKRDHMVLL
210	222	DPNEKRDHMVLLE
216	224	DHMVLLEFV
222	238	EFVTAAGITLGMDELYK

(P+AP) = proline modified with aminophenol

Figure S16. (a) To identify the residue on proline-GFP that was modified via the oxidative coupling, the protein was first treated with 0.1 mM aminophenol in the presence of 5 mM ferricyanidein phosphate buffer at pH 7.5. The modified protein was subjected to trypsin digest and LC-MS/MS analysis. (b) Proline-GFP sequence with residues observed in the LC-MS/MS analysis bolded. The modified residue is highlighted in pink. (c) The listed peptides were observed during LC-MS/MS analysis. Only the longest, unique fragments observed are shown, except for those fragments that included the modification. 91% sequence coverage was observed.

S.21 Optimization of reaction conditions on protein substrates

a



b

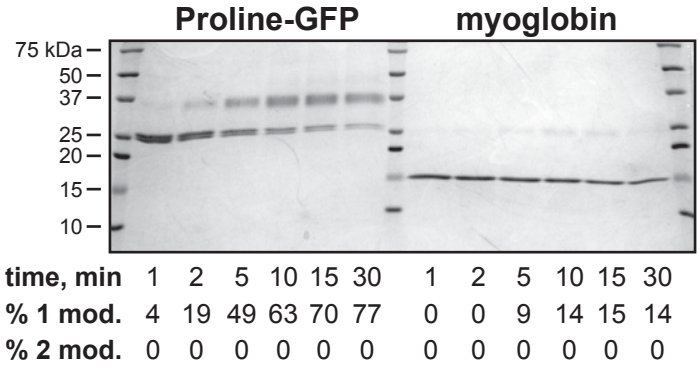
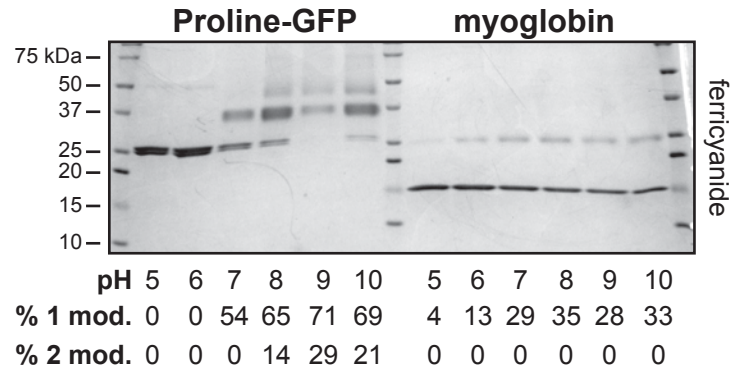


Figure S17. The buffer compatibility and time course of the reaction were tested on protein substrates. The reactivity was assayed on a protein with the optimized proline terminus (Proline-GFP) and one with its native terminus (myoglobin). Reactions were run with 20 μM protein, 200 μM *o*-aminophenol PEG and 5 mM ferricyanide at pH 7.5 (a) Several buffer salts were tested for their effect on the reactivity. (b) The reaction was monitored at various time points to determine the course of the reaction. Aliquots of the reaction were taken out at the indicated time point and quenched by the addition of loading buffer.

a



b

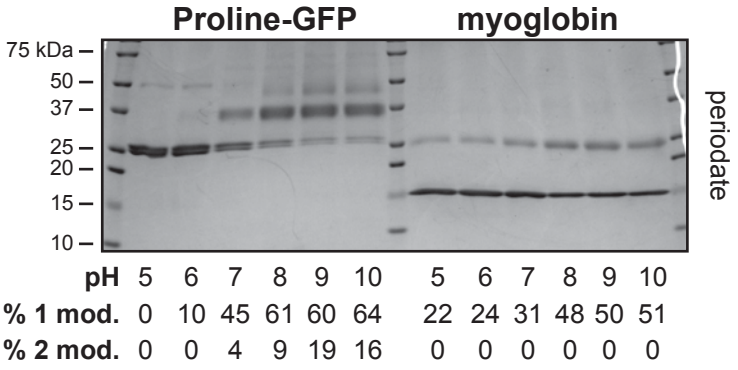


Figure S18. The effect of pH on reactivity and selectivity of the reaction was assayed by SDS-PAGE. The reactivity was assayed on a protein with the optimized proline terminus (Proline-GFP) and one with its native terminus (myoglobin). Reactions were run with 20 μM protein, 200 μM *o*-aminophenol PEG and 5 mM oxidant and were quenched by the addition of loading buffer after 30 min. Both (a) ferricyanide and (b) periodate were tested as oxidants for this reaction.

S.22 Wide mass range and ion series for Figure 4b

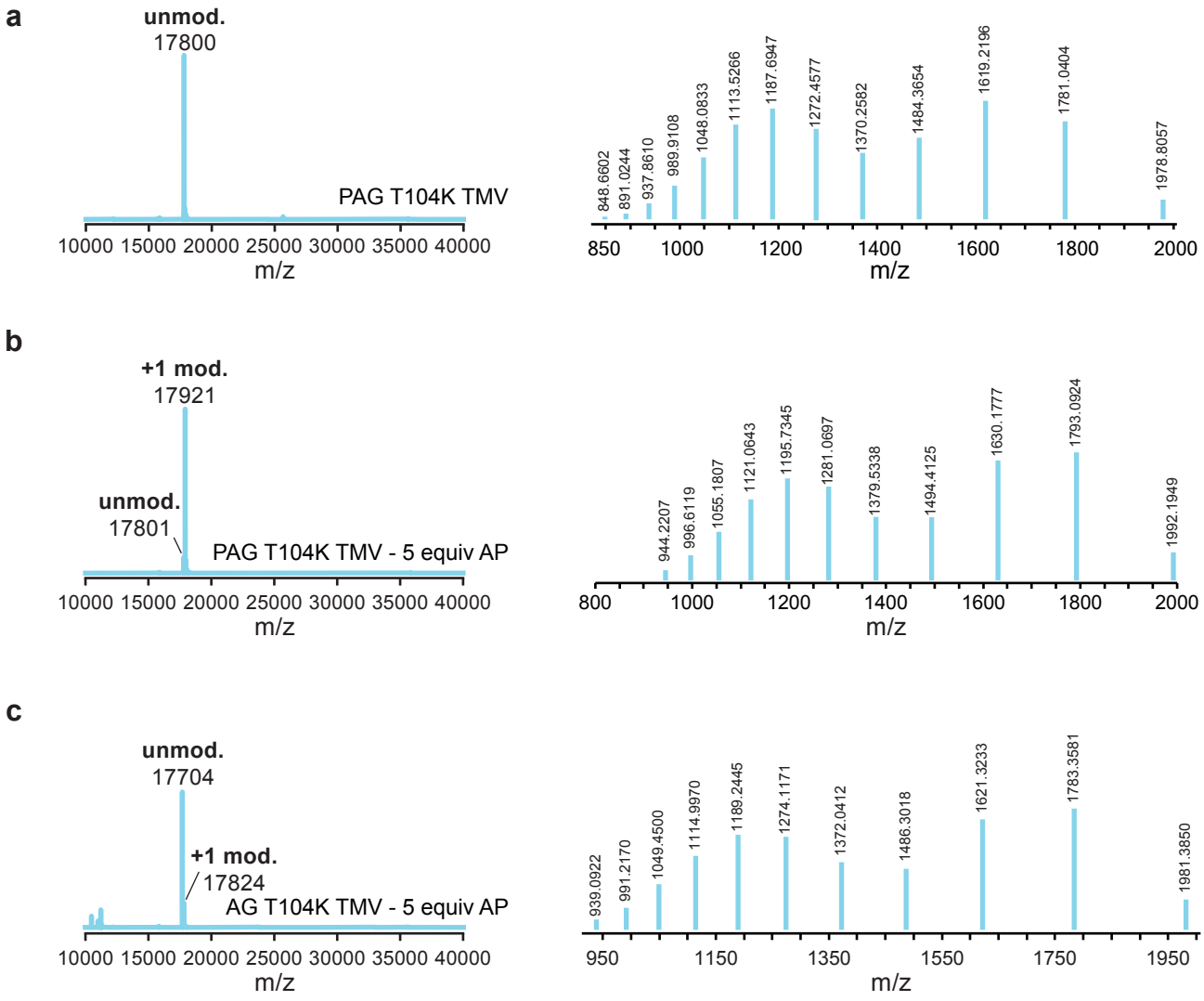


Figure S19. Wide mass range and ion series for (a) unmodified PAG T104K TMV, (b) PAG T104K TMV reacted with 5 equiv of 2-amino-*p*-cresol in the presence of ferricyanide and (c) AG T104K TMV reacted with 5 equiv of 2-amino-*p*-cresol in the presence of ferricyanide.

S.23 Cysteine reactivity with o-aminophenols

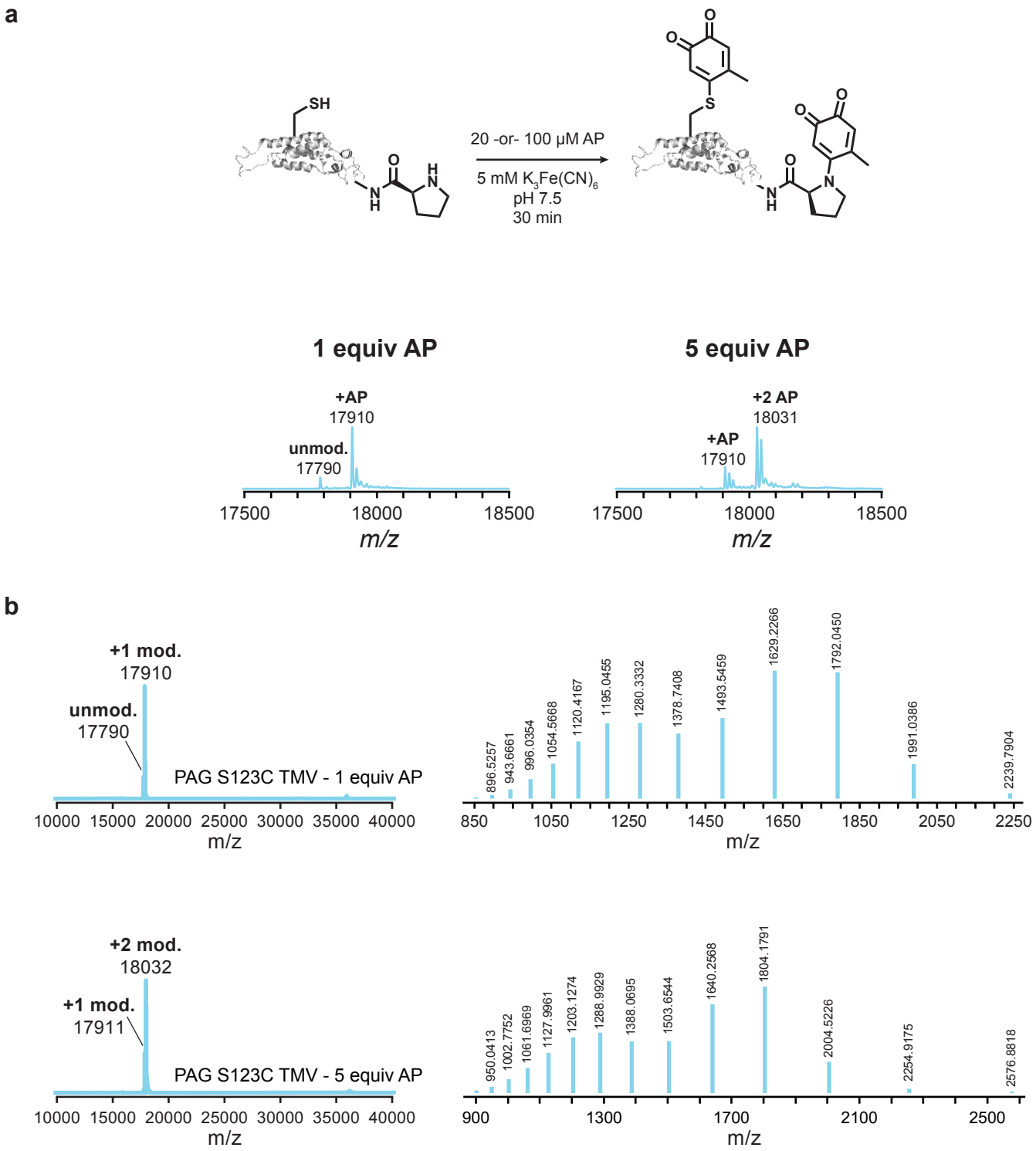


Figure S20. Compatibility with cysteine residues. (a) PAG S123C TMV was reacted with either 1 or 5 equiv of 2-amino-*p*-cresol in the presence of ferricyanide and analyzed by LC-MS. (b) Wide mass range and ion series for the traces shown in (a).

S.24 Wide mass range and ion series for Figure 5

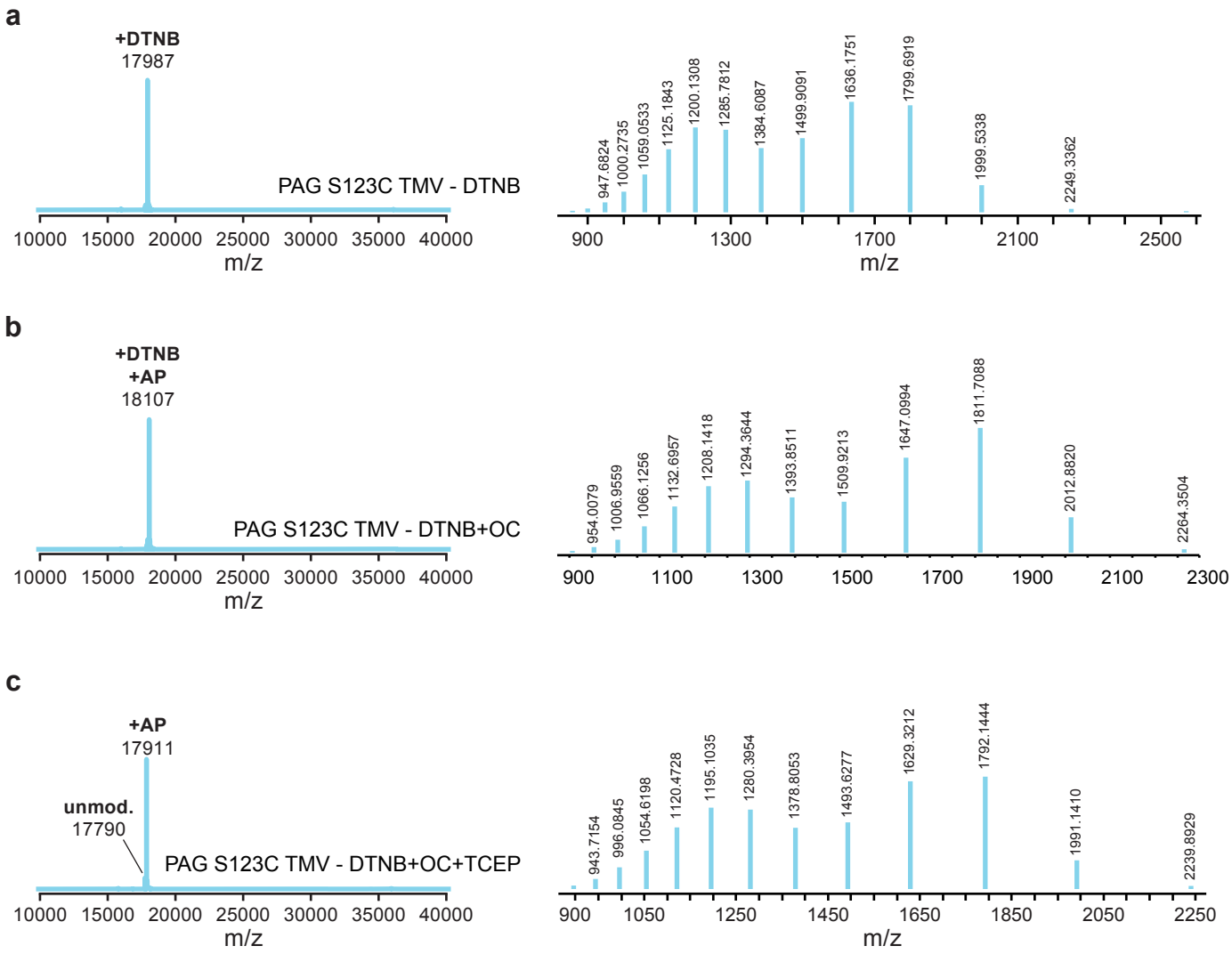


Figure S21. Wide mass range and ion series for (a) PAG S123C TMV modified with Ellman's reagent (DTNB) (b) PAG S123C TMV modified with DTNB and the oxidative coupling reaction (OC) and (c) PAG S123C TMV modified with DTNB, OC, and then reduced with TCEP.

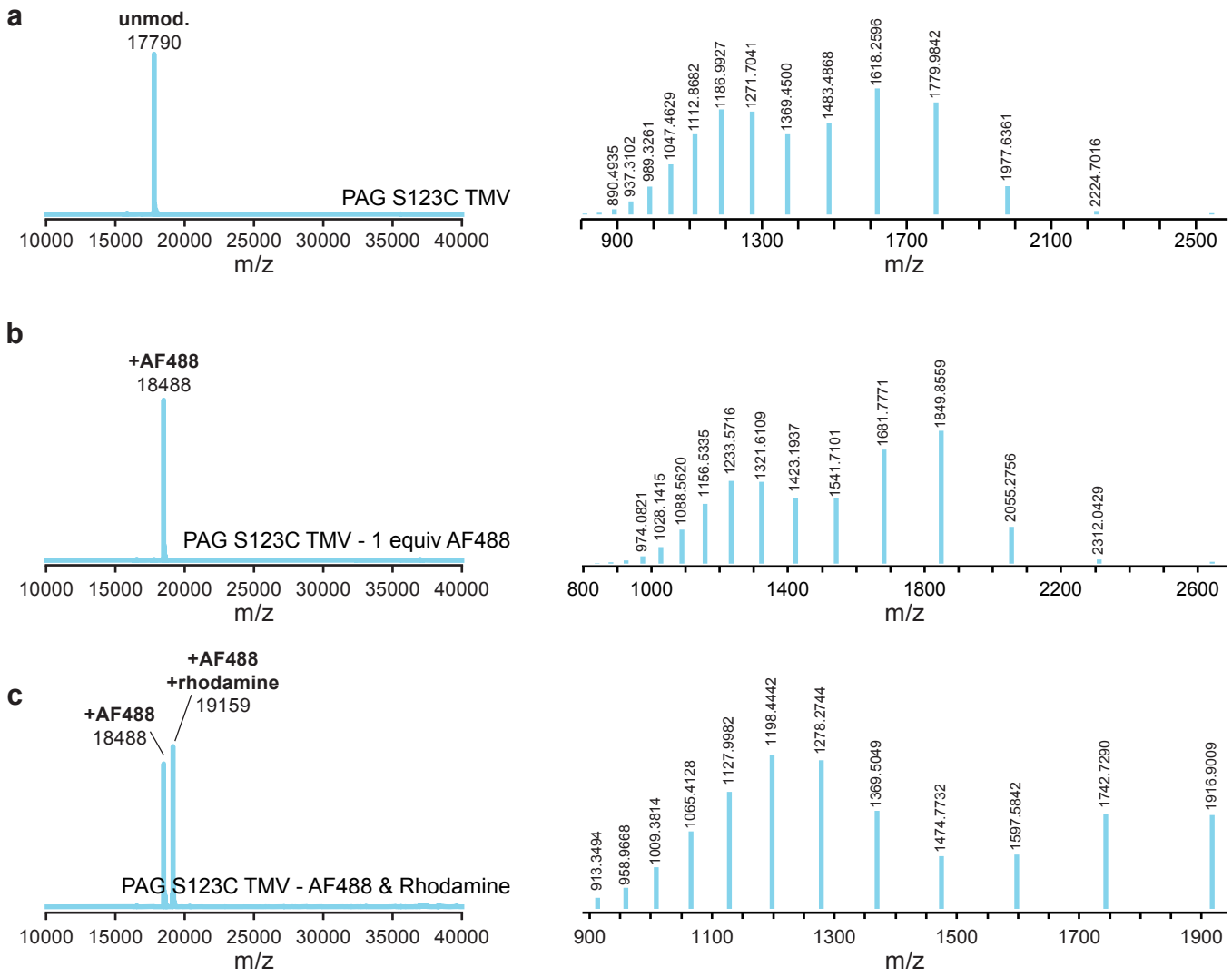


Figure S22. Wide mass range and ion series for (a) unmodified PAG S123C TMV, (b) PAG S123C TMV modified with Alexa Fluor 488 maleimide (AF488), and (c) PAG S123C TMV modified with AF488 followed by oxidative coupling with a rhodamine aminophenol (Rhodamine).