Supporting Information

Title: Pyrophosphorylation *via* Selective Phosphoprotein Derivatization

Alan M. Marmelstein,[a,b] Jeremy A. M. Morgan,[a] Martin Penkert,[a,c] Daniel T. Rogerson,[d] Jason W. Chin,[d] Eberhard Krause,[a] and Dorothea Fiedler*[a,c]

[a]Leibniz-Forschungsinstitut für Molekulare Pharmakologie, Robert-Rössle Str. 10, 13125 Berlin, Germany; [b]Department of Chemistry, Princeton University, Washington Road, Princeton, New Jersey 08544, United States; [c]Institut für Chemie, Humboldt Universität zu Berlin, Brook-Taylor-Str. 2, 12489 Berlin, Germany; [d]Medical Research Council Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, UK

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I. Supporting Figures

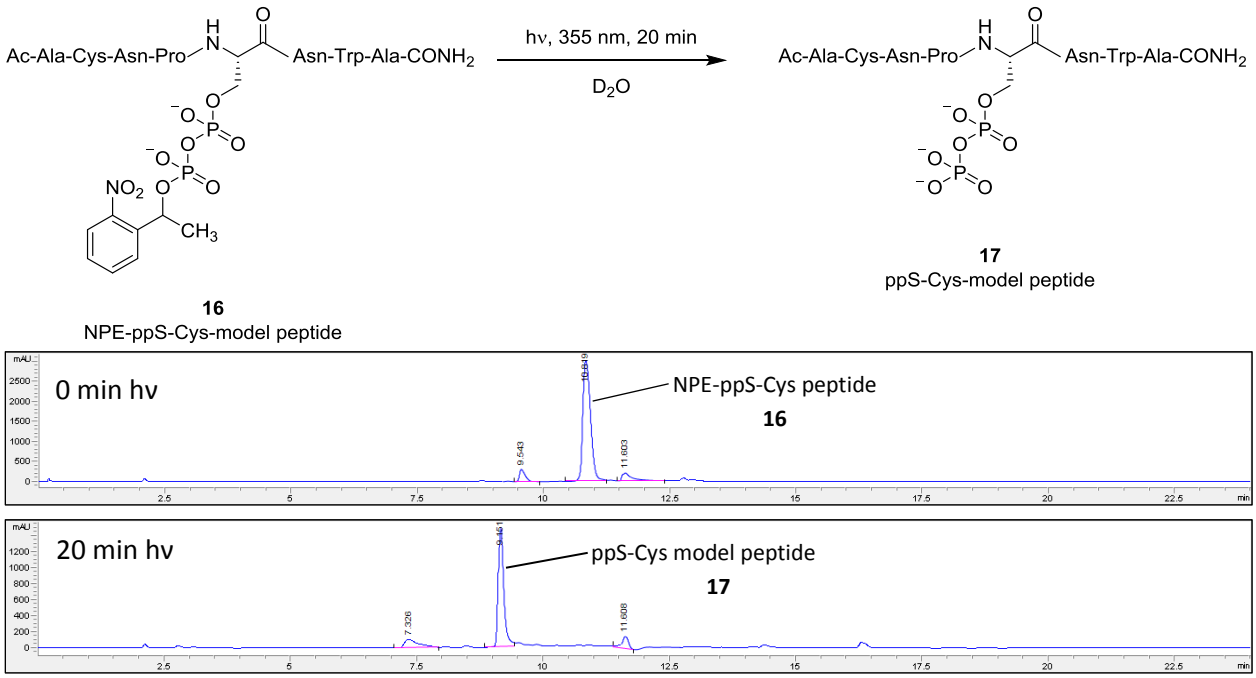


Figure S1. HPLC **Method B** (UV = 274 nm) monitoring of NPE-ppS-Cys model peptide **16** photo-deprotection.

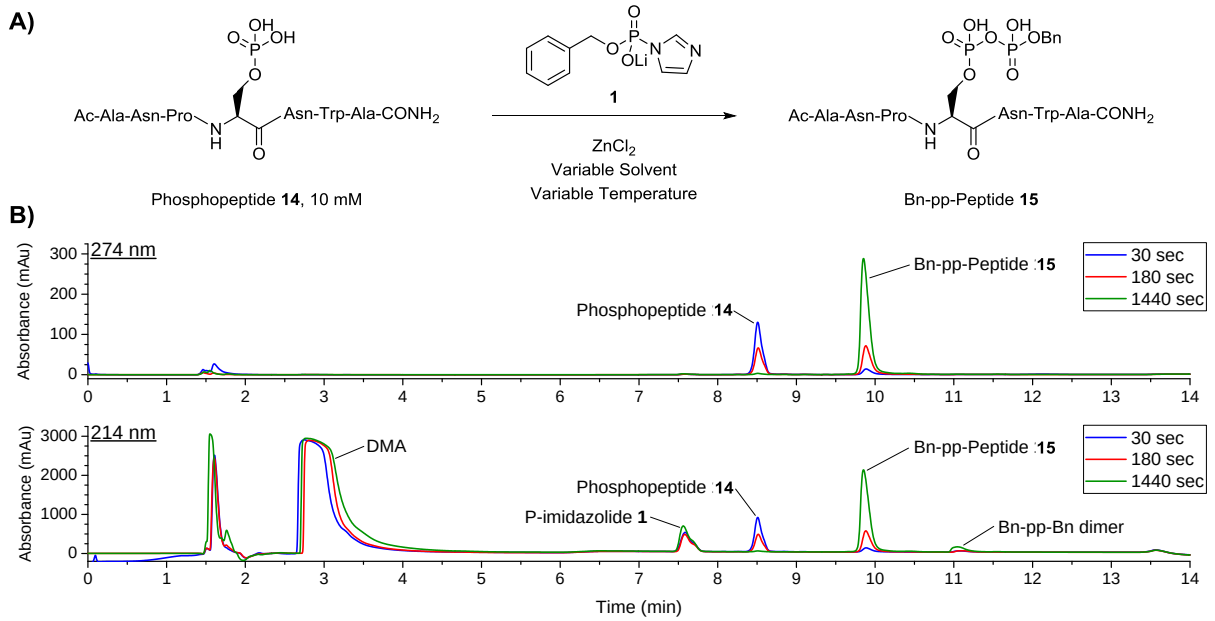


Figure S2. Model pyrophosphorylation reaction for kinetic characterization. A) Reaction of model phosphopeptide **14** with phosphorimidazolide **1**. B) Typical HPLC chromatograms of time-points at the beginning, middle, and end of the reaction, monitored at 274 nm and 214 nm. Reaction conditions: 10 mM model phosphopeptide **14**, 30 mM P-imidazolide reagent **1**, and 80 mM $ZnCl_2$ at 45 ˚C in 1:9 H_2O:DMA solvent.

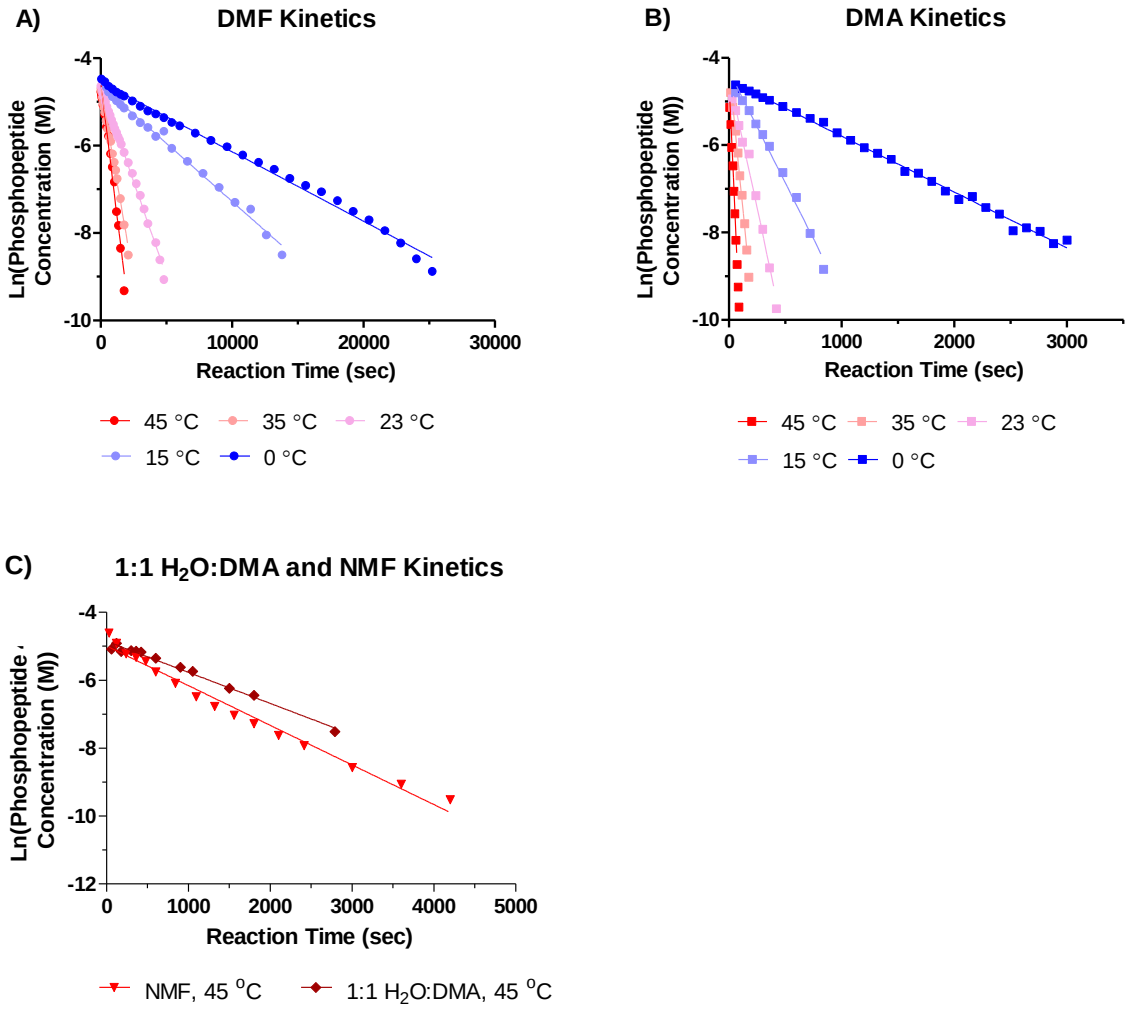
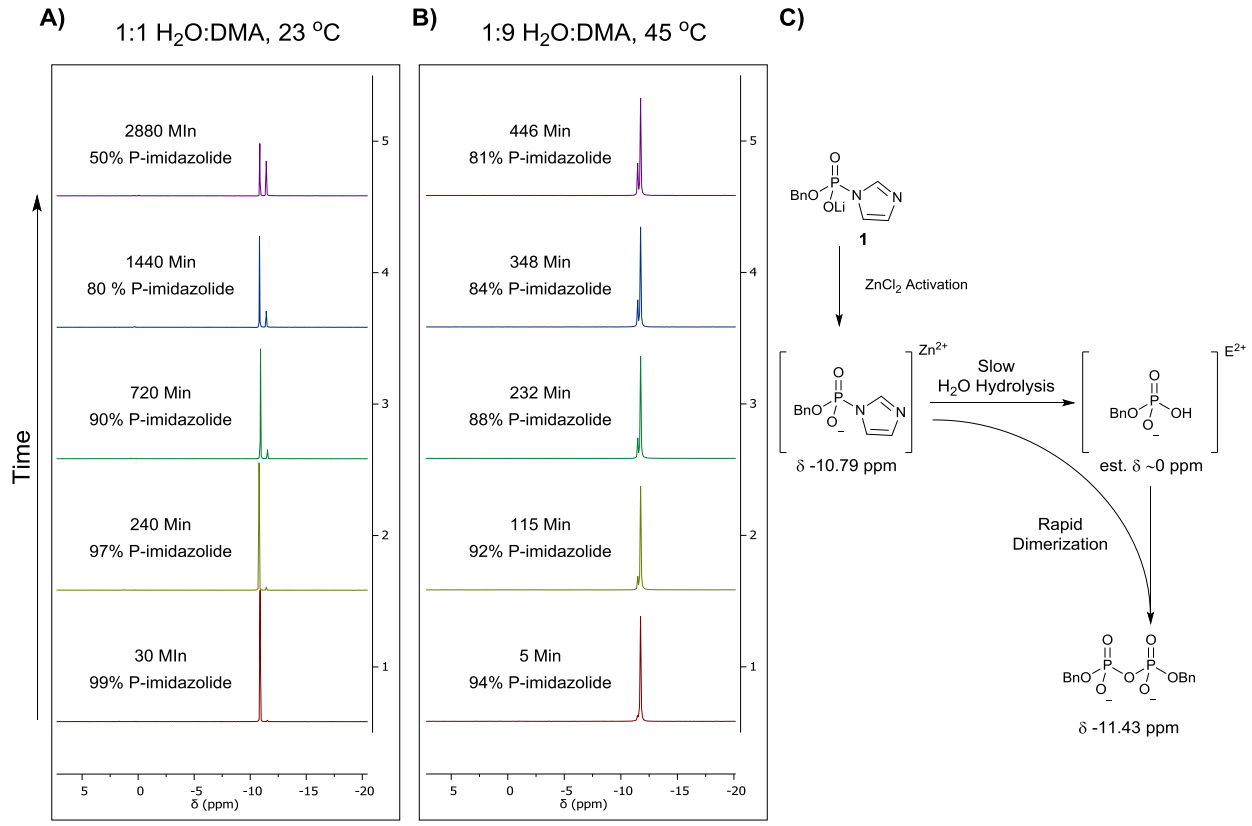
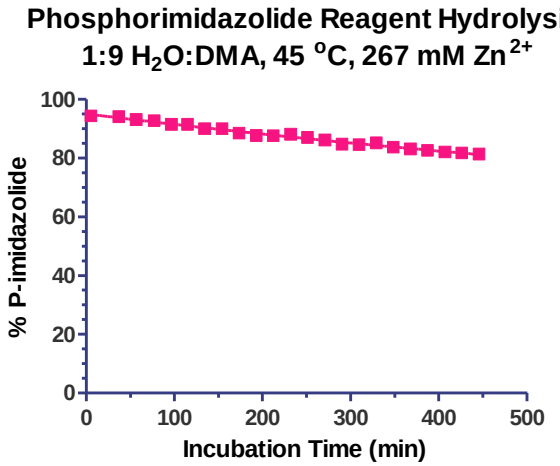


Figure S3. Plots of pseudo-first order kinetics data from the model pyrophosphorylation reaction in **Figure 2a**. Reaction conditions: 100 mM P-imidazolide **1**, 10 mM Phosphopeptide **14**, 267 mM $ZnCl_2$ in A) DMF, B) DMA, and C) 1:1 H_2O:DMA and NMF, solvent respectively at various temperatures. Reactions in DMA are much more rapid than those in DMF.



D)



One phase decay	
Best-fit values	
Y0	95.11
Plateau	51.44
K	0.0008590
Half Life	807.0
Tau	1164
Span	43.67

Figure S4. Monitoring hydrolysis of P-imidazolide reagent **1**. A) Hydrolysis and dimerization in 1:1 H_2O:DMA at room temperature. 50 mM reagent **1** with 133 mM $ZnCl_2$. Reagent has a half-life of 48 hours. B) Hydrolysis and dimerization in 1:9 H_2O:DMA at 45 ˚C. 100 mM reagent **1** with 267 mM $ZnCl_2$. Reagent has a half-life of 13.5 hours according to a one-phase decay fit of the data (D) Scheme depicting likely degradation pathway of reagent **1**. After hydrolysis of the Zn^{2+}-activated reagent by water, the resulting phosphate monoester (**18**) reacts rapidly with the remaining Zn^{2+}-activated reagent to form a dibenzylpyrophosphate dimer which can be observed

at -11.4 ppm. D) Graph of P-imidazolide reagent **1** hydrolysis data in 1:9 H_2O:DMA at 45 ˚C with one-phase decay fit and corresponding parameters. The amount of P-imidazolide in solution relative to the degradation product was determined by monitoring *via* ^{31}P NMR and integrating the P-imidazolide and hydrolysis product peaks (B).

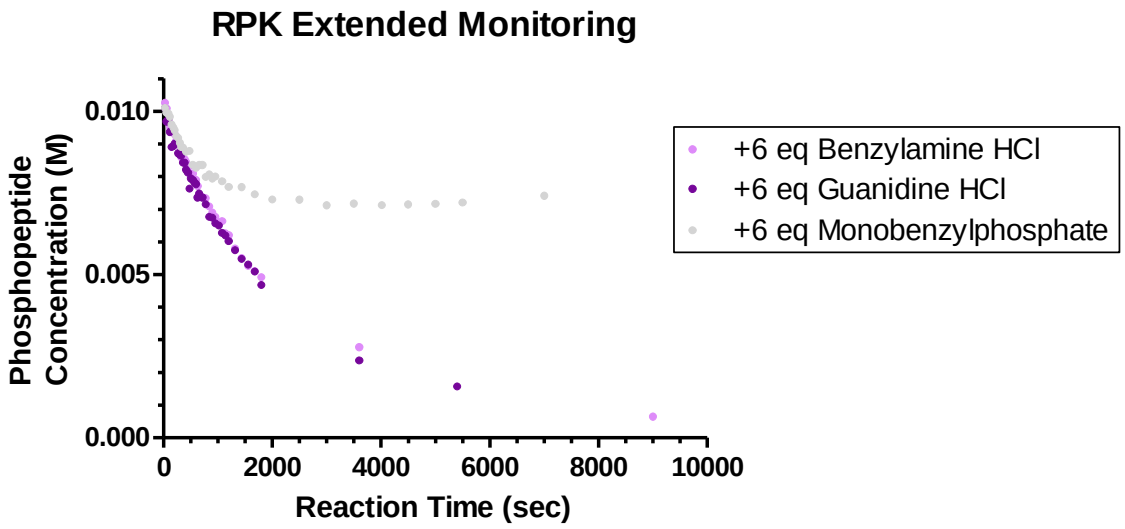


Figure S5. Extended monitoring of RPKA reactions with added benzylamine HCl, guanidine HCl, or monobenzylphosphate (**18**). The reaction with added monobenzylphosphate stalls at 27-30% conversion while the reactions with amine or guanidine additives progress to high conversion.

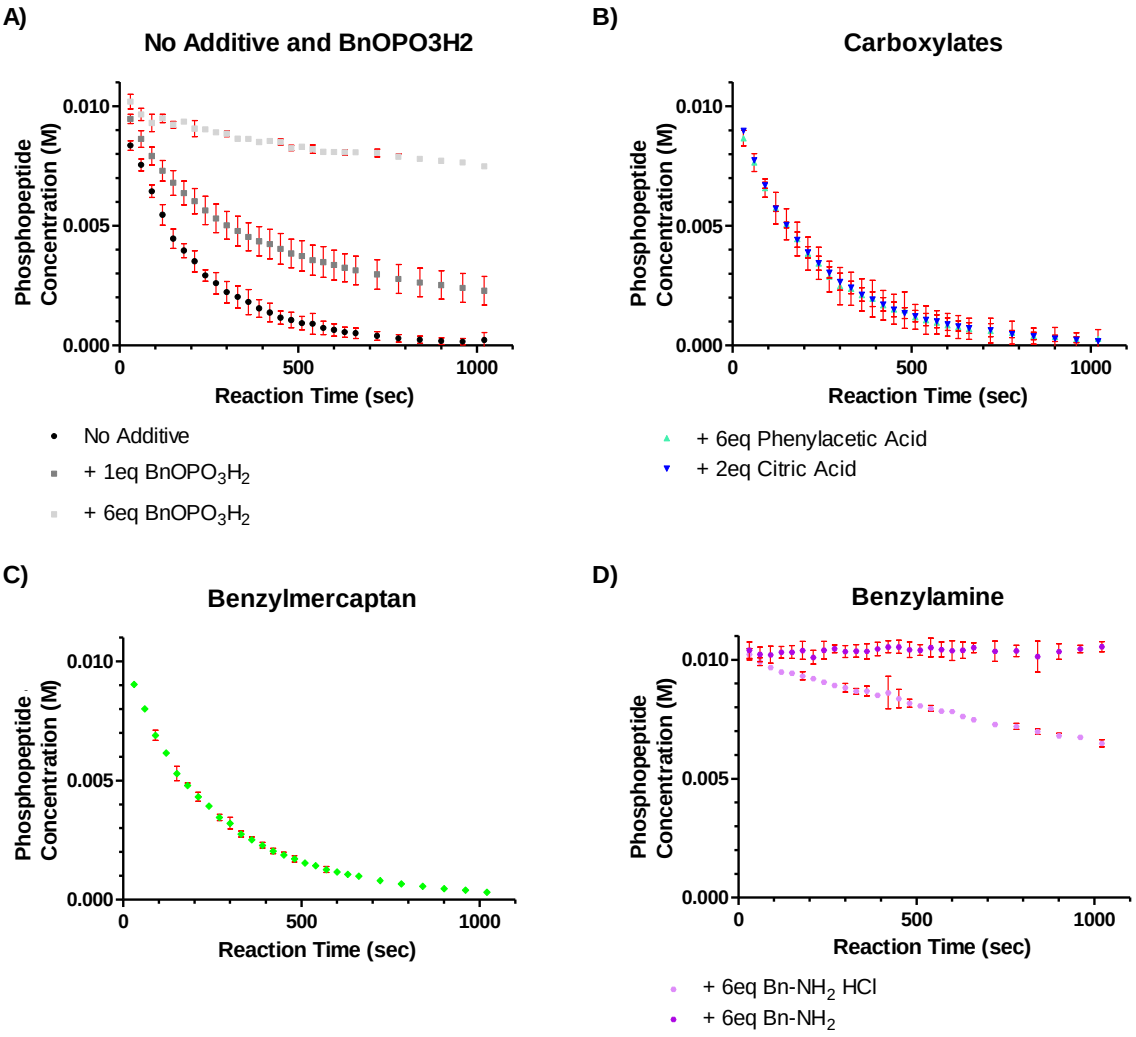
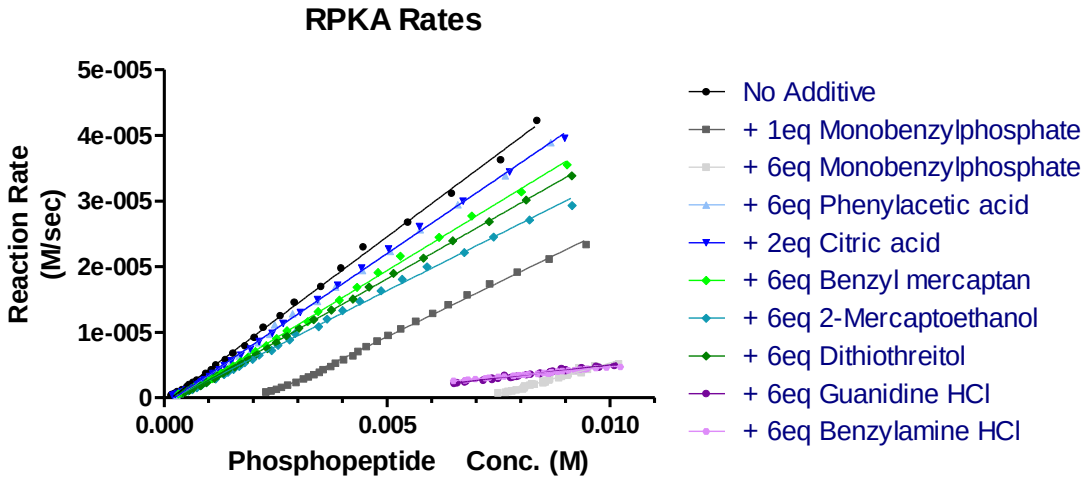


Figure S6. Reaction progress kinetics data presented in **Figure 2** with standard deviation error bars added for data points that represent averages from three replicate reactions. Reaction being monitored is depicted in **Figure 2a**. Reaction conditions: 30 mM P-imidazolide **1**, 10 mM phosphopeptide **14**, 80 mM $ZnCl_2$, in 1:9 H_2O:DMA at 45 ˚C.

A)



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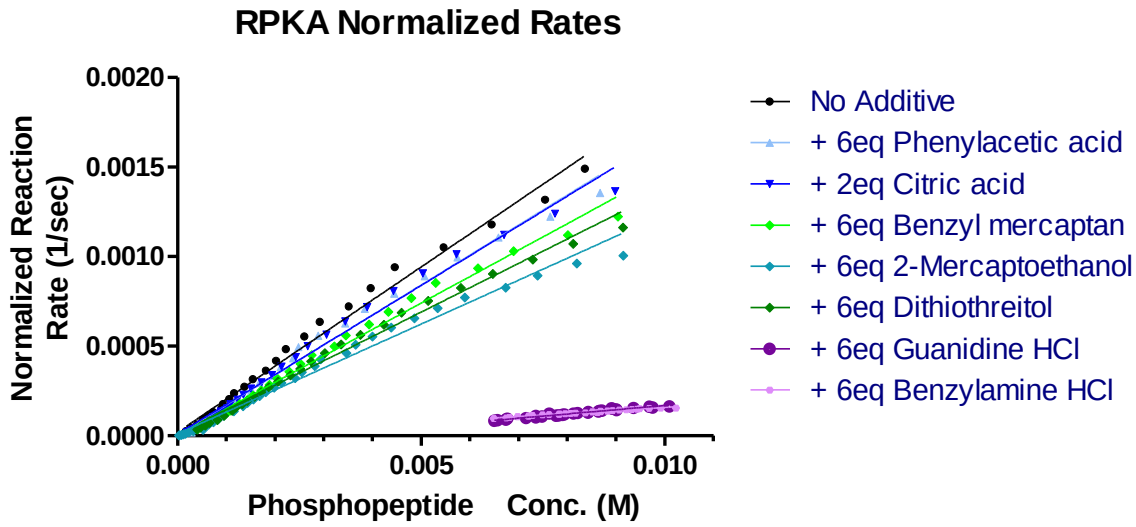


Figure S7. Collated and processed RPKA data. See Kinetics Experimental Protocols and Data Analysis Section (VII) for details of data processing. A) Instantaneous reaction rates calculated using the derivative (**Equation 2**) of the one-phase decay exponential fit to concentration vs. time data (**Figure 2**) and plotted here against phosphopeptide **14** concentration. The slope of each line is the observed rate constant k_{obs} for each reaction in sec^{-1}. B) Instantaneous reaction rates normalized to concentrations of Phosphorimidazolide reagent **1** at each time-point as calculated using **Equation 3**. The slope of each line corresponds to the second order rate constant k for each reaction in $M^{-1}sec^{-1}$.

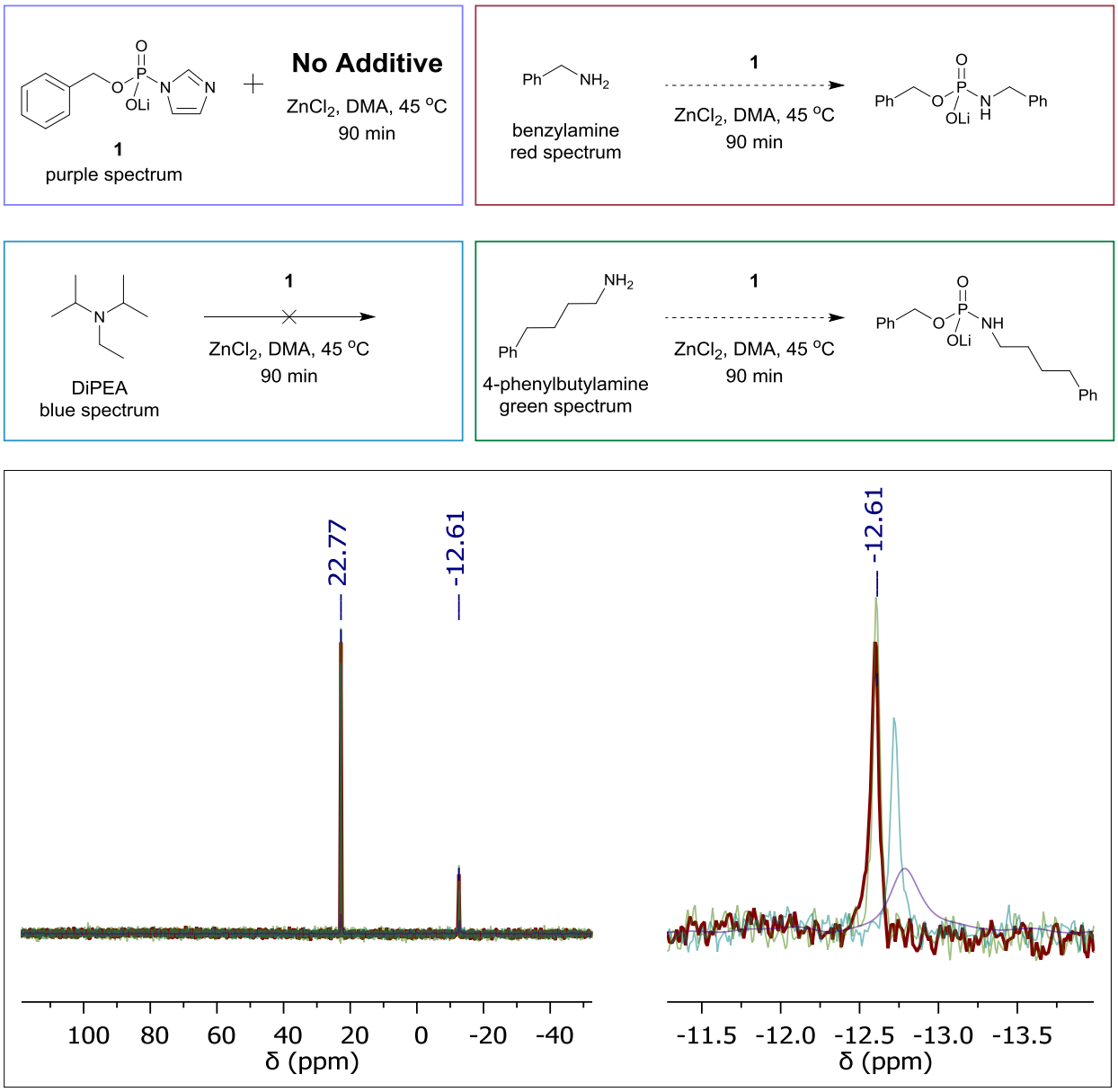


Figure S8. Phosphorimidazolide reagent **1** (80 mM) activated with Zn^{2+} (213 mM) was incubated with various amines in neat DMA at 45 ˚C and submitted directly to ^{31}P NMR analysis. NMR spectra were taken at 203 MHz, in non-deuterated DMA. Each sample had a sealed capillary containing a solution of $P(CH_3)_4Br$ (22.77 ppm) in D_2O for calibration and locking. Purple = no additive; Blue = + 0.5 equivalents DiPEA (non-nucleophilic control); Green = + 0.5 equivalents 4-phenylbutylamine; Red = + 0.5 equivalents benzylamine.

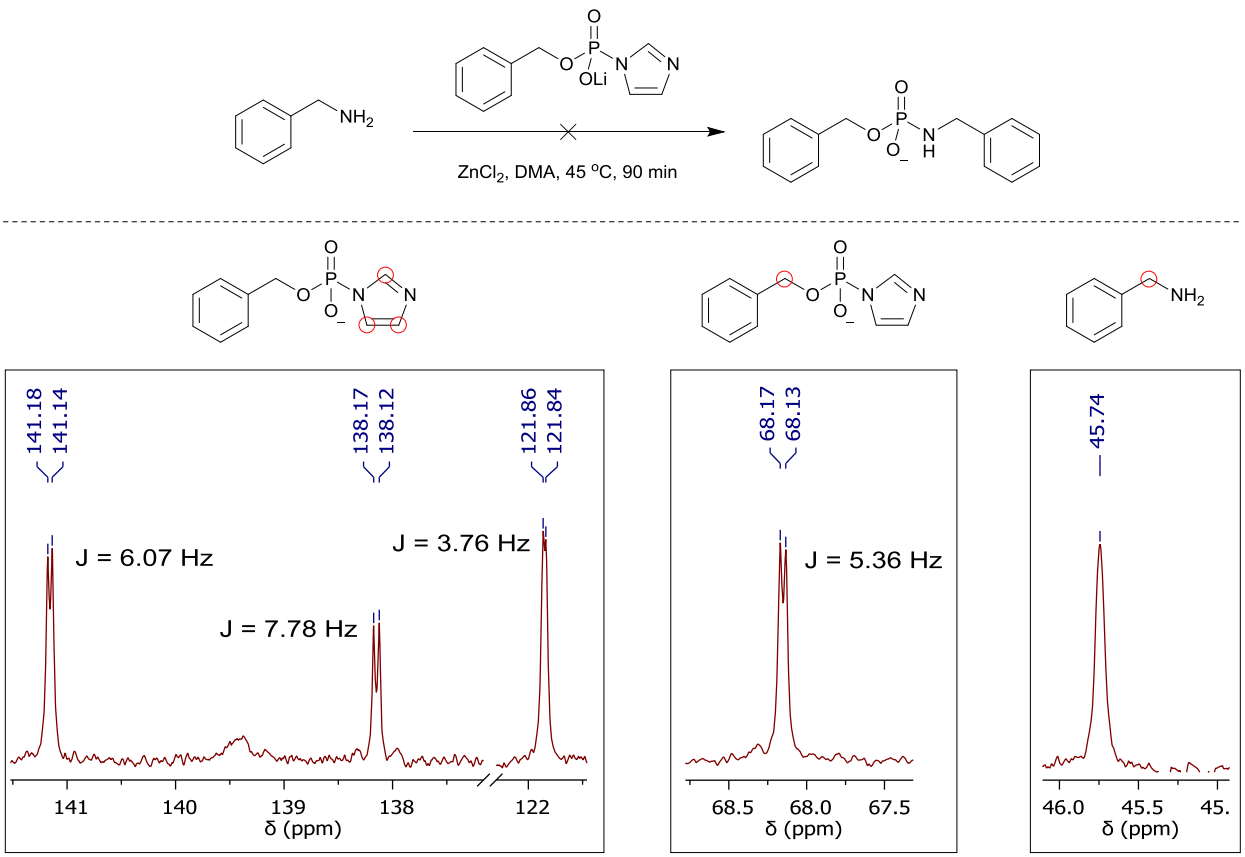


Figure S9. Details of ^{13}C NMR spectrum of Zn^{2+}-activated phosphorimidazolide reagent **1** treated with benzylamine in neat DMA and heated to 45 ˚C for 90 minutes. NMR spectrum was taken in neat DMA and sample tube was equipped with a sealed capillary containing a solution of $P(CH_3)_4Br$ (22.77 ppm) in D_2O for calibration and locking. The *O*-benzylic carbon of benzyl alcohol appears at 64.7 ppm[1] while the *N*-benzylic carbon of benzylamine appears upfield, at 46.4 ppm[2] in $CDCl_3$, allowing the two species to be readily distinguished. Splitting of the three imidazole carbons by phosphorus indicates that the intact phosphorimidazolide is the major species.

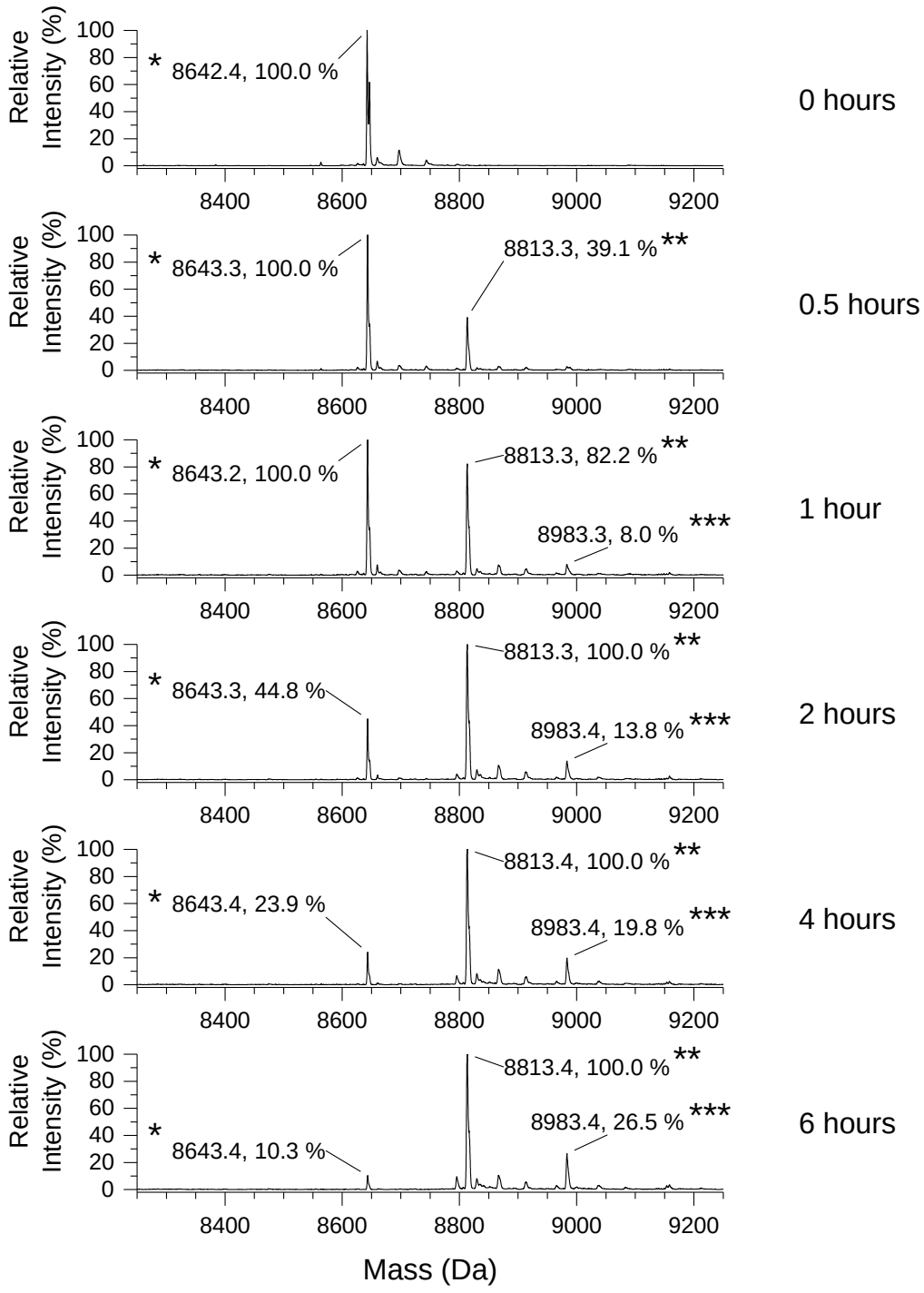


Figure S10. * = Ub-S65pS, ** = Ub-S65pS + $BnOPO_2$, *** = Ub-S65pS + 2x $BnOPO_2$. ESI-MS monitoring of pyrophosphorylation of Ub-S65pS with reagent **1** (bottom panel). Mass resolution of 0.1 Da. Error 10 ppm.

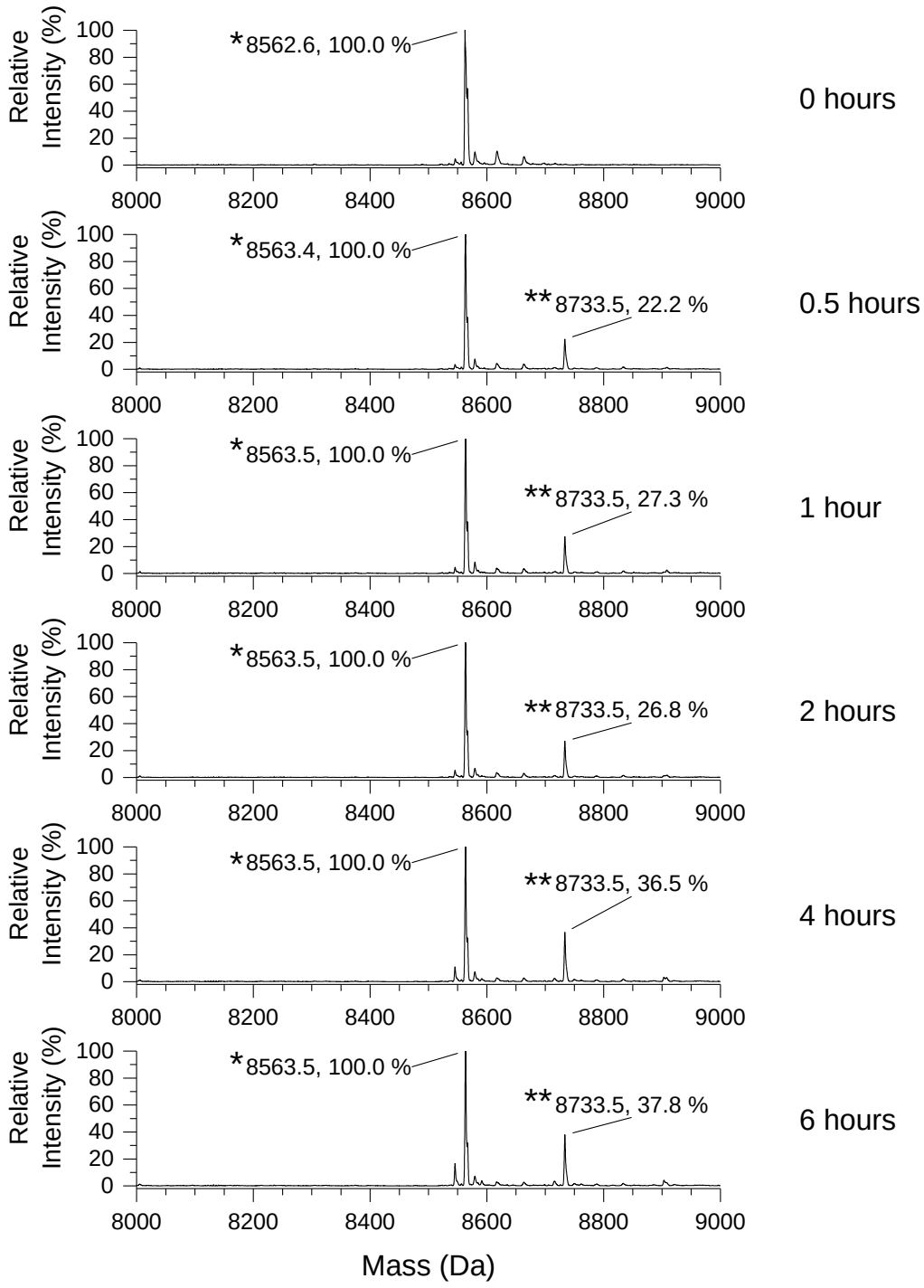


Figure S11. * = Ub-wt, ** = Ub-wt + $BnOPO_2$. ESI-MS monitoring of Ub-wt under pyrophosphorylation reaction conditions with reagent 1 (bottom panel). Mass resolution of 0.1 Da. Error 10 ppm.

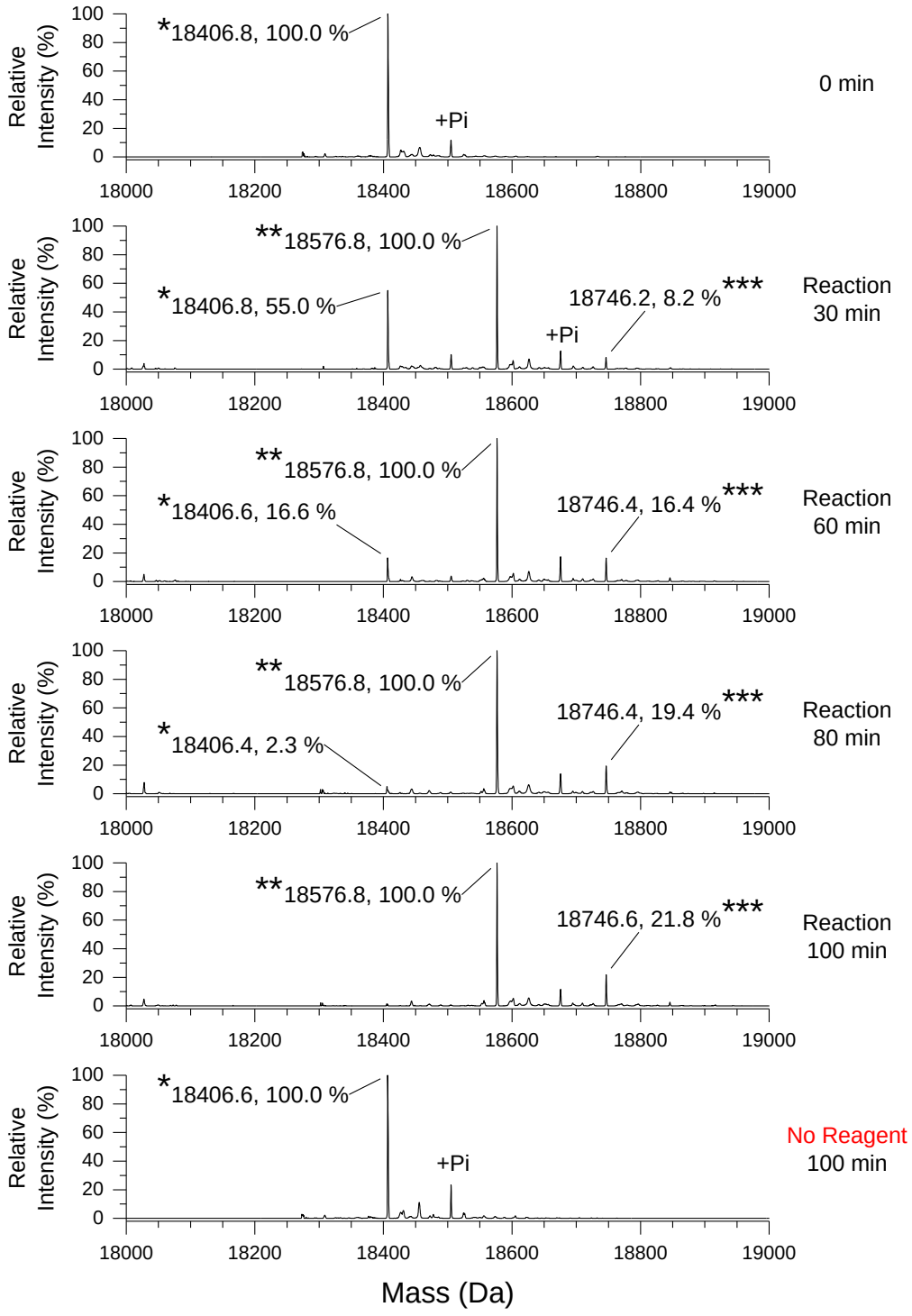


Figure S12. * = Myo-D127pS, ** = Myo-D127pS + $BnOPO_2$, *** = Myo-D127pS +2x $BnOPO_2$. ESI-MS timepoints of Myo-D127pS pyrophosphorylated with reagent **1**. The reagent negative control was subject to the reaction conditions (306 mM $ZnCl_2$, 1:9 H_2O:DMA, 45 °C) for 100 minutes without **1** and worked up using the standard protocol.

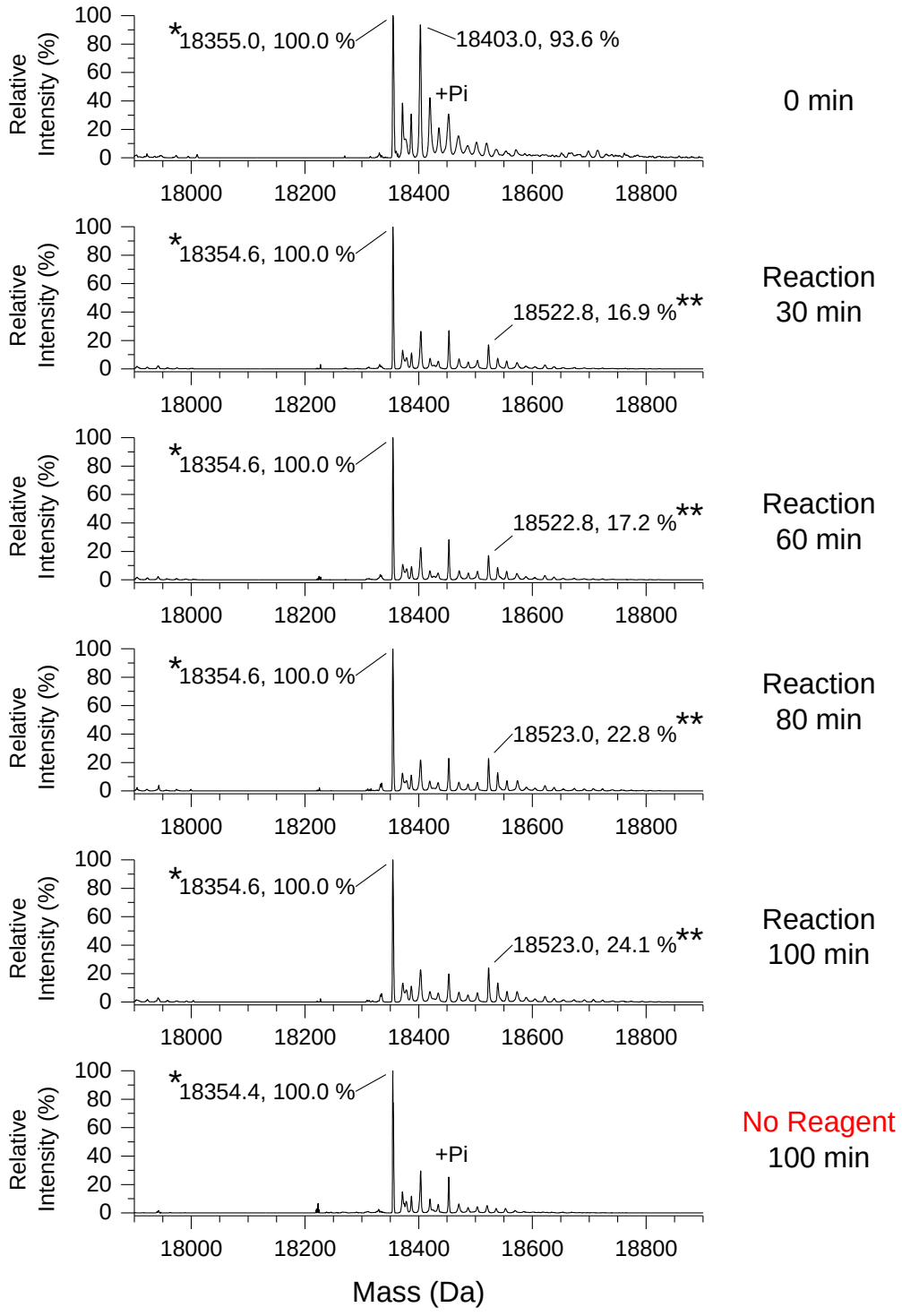


Figure S13. * = Myo-wt, ** = Myo-wt + $BnOPO_2$. ESI-MS timepoints of wild type myoglobin pyrophosphorylated with reagent **1**. The reagent negative control was subject to reaction conditions (306 mM $ZnCl_2$, 1:9 H_2O:DMA, 45 °C) for 100 minutes without **1** and worked up using the standard protocol.

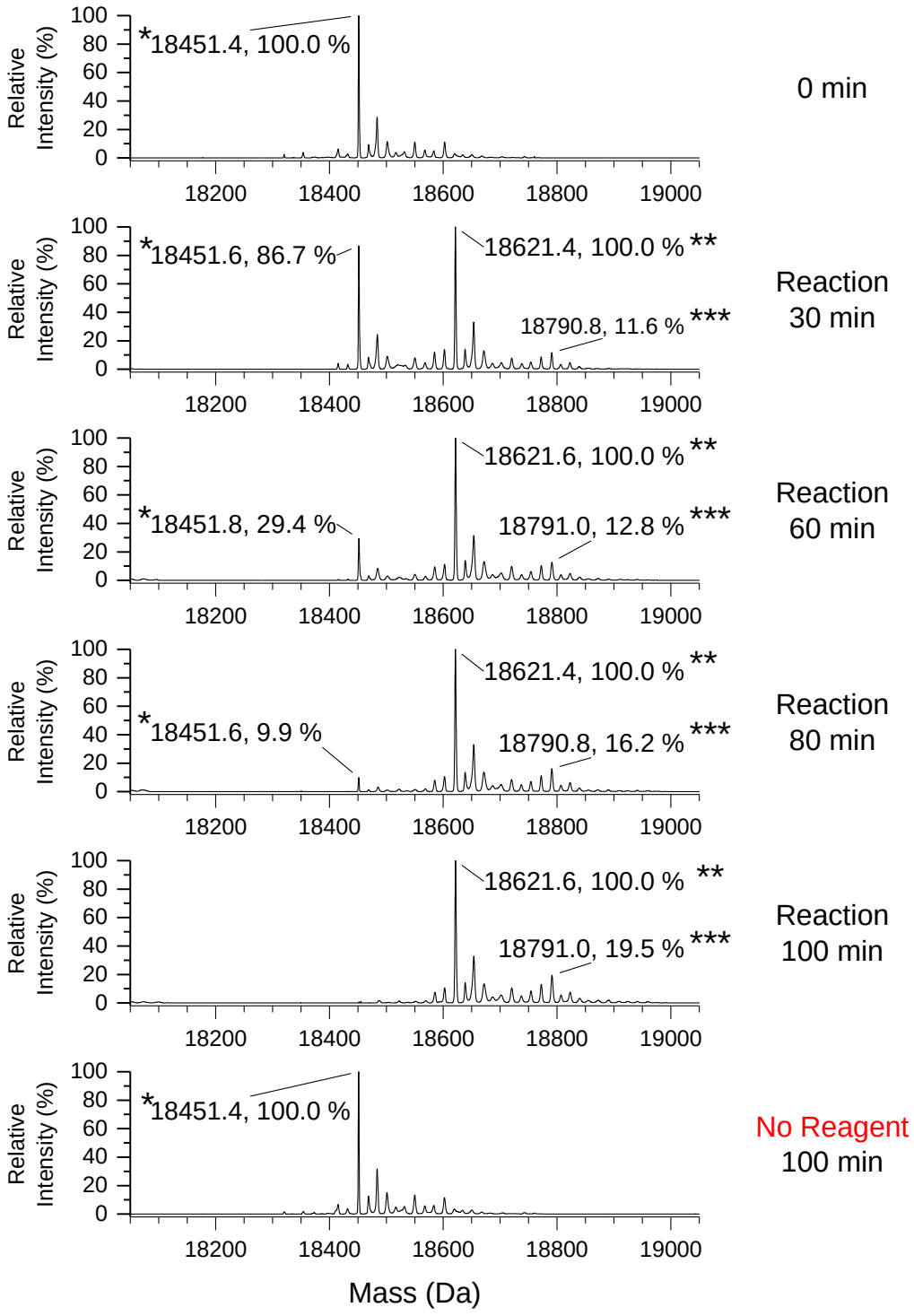


Figure S14. * = Myo-D127pS-G154C, ** = Myo-D127pS-G154C + $BnOPO_2$, *** = Myo-D127pS-G154C +2x $BnOPO_2$. ESI-MS timepoints of Myo-D127pS-G154C pyrophosphorylated with reagent **1**. The reagent negative control was subject to reaction conditions (306 mM $ZnCl_2$, 1:9 H_2O:DMA, 45 °C) for 100 minutes without **1** and worked up using the standard protocol.

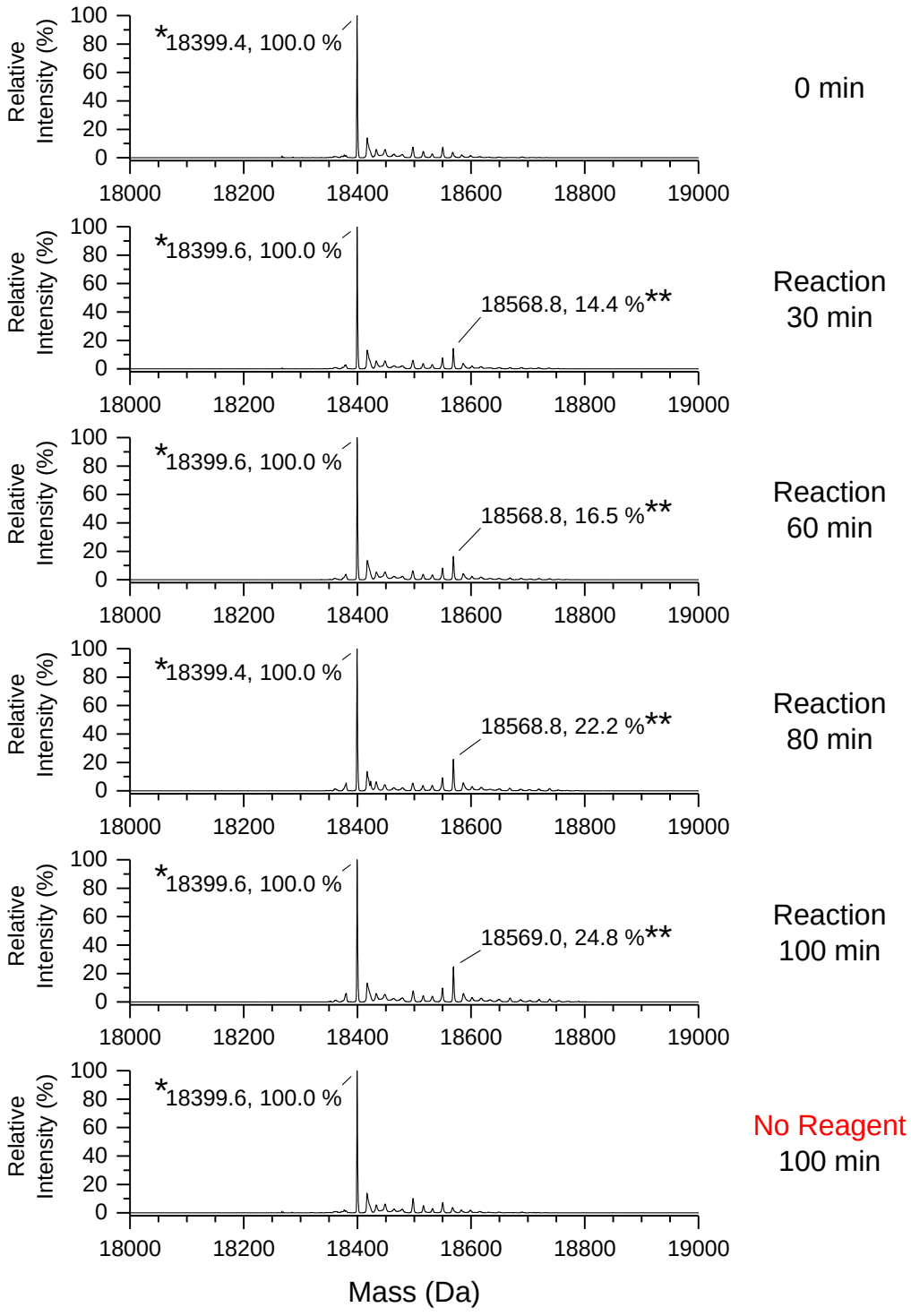


Figure S15. * = Myo-G154C, ** = Myo-G154C + $BnOPO_2$. ESI-MS timepoints of Myo-G154C subject to pyrophosphorylation reaction conditions with reagent **1**. The reagent negative control was subject to reaction conditions (306 mM $ZnCl_2$, 1:9 H_2O:DMA, 45 °C) for 100 minutes without **1** and worked up using the standard protocol.

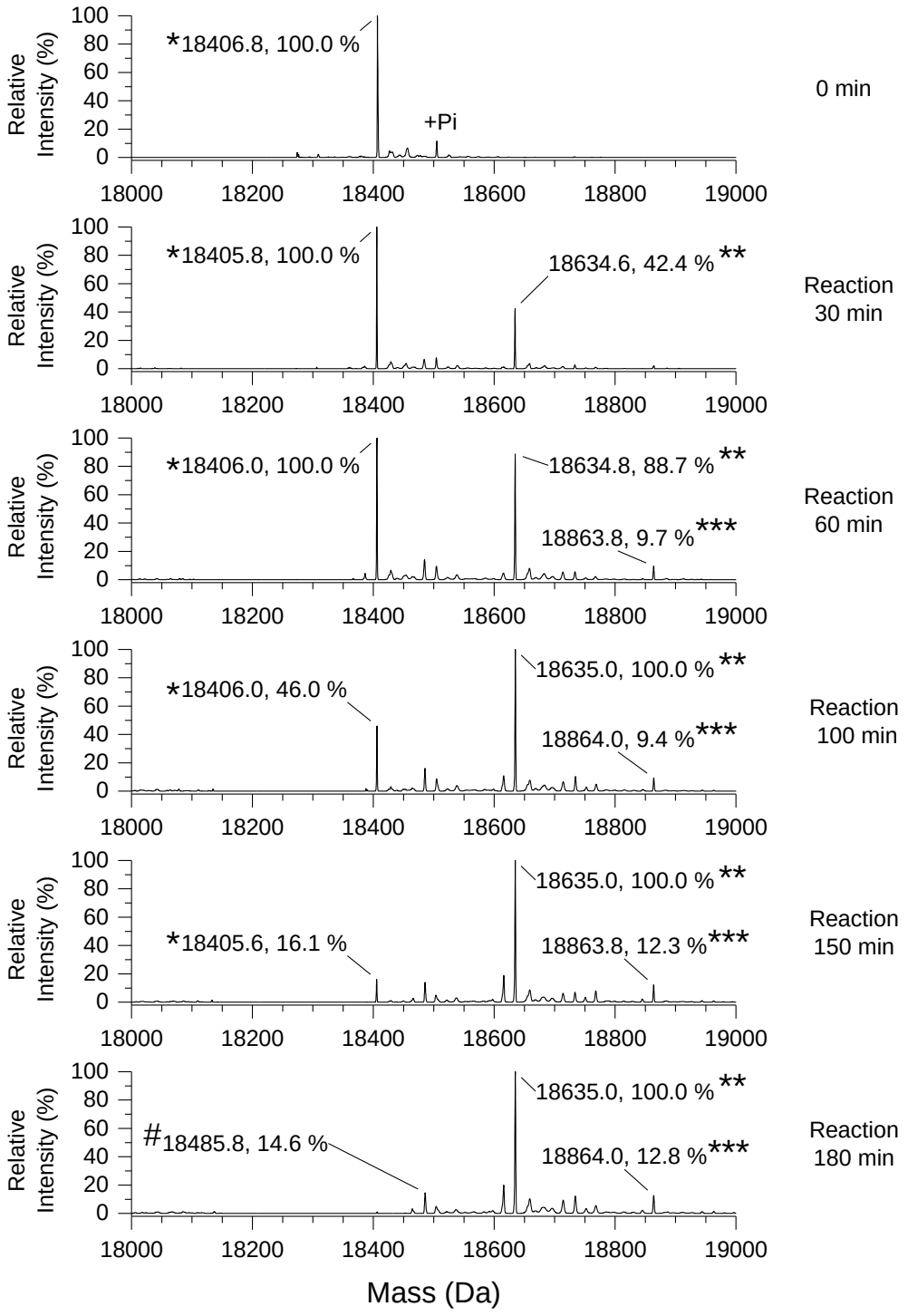


Figure S16. * = Myo-D127pS, ** = Myo-D127S + NPE-OPO_2, *** = Myo-D127pS + 2x NPE-OPO_2, # = Myo-127ppS. ESI-MS timepoints of Myo-D127pS pyrophosphorylated with reagent **2**. A small amount of free pyrophosphorylated myoglobin can be observed (Myo-D127ppS; expected mass: 18487.02 Da).

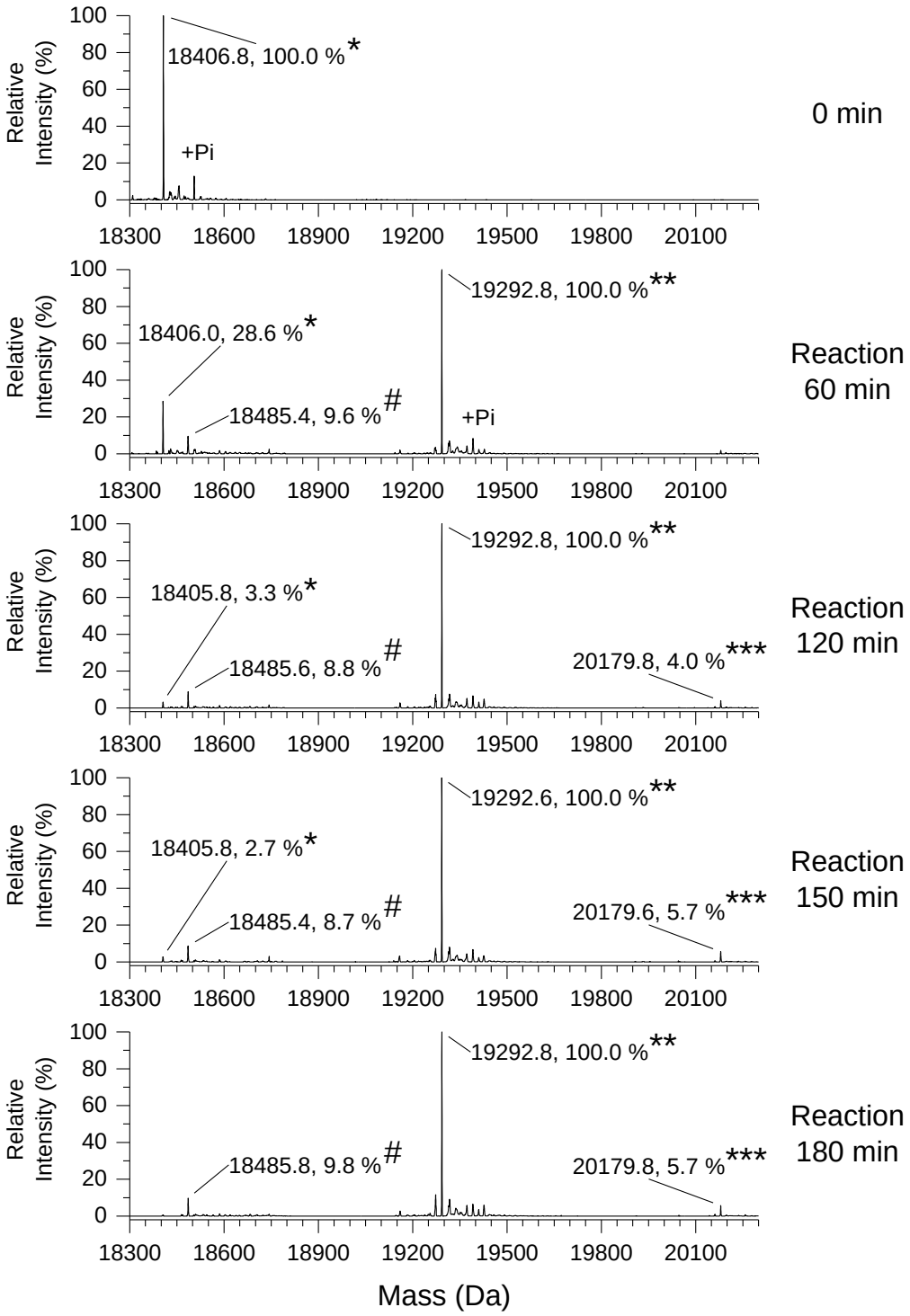


Figure S17. * = Myo-D127pS, ** = Myo-D127pS + (**3**-Im), *** = Myo-D127pS +2x (**3**-Im), # = Myo-ppS127. ESI-MS timepoints of Myo-D127pS pyrophosphorylated with reagent **3**. A small amount of free pyrophosphorylated myoglobin can be observed (Myo-D127ppS; expected mass: 18487.02 Da).

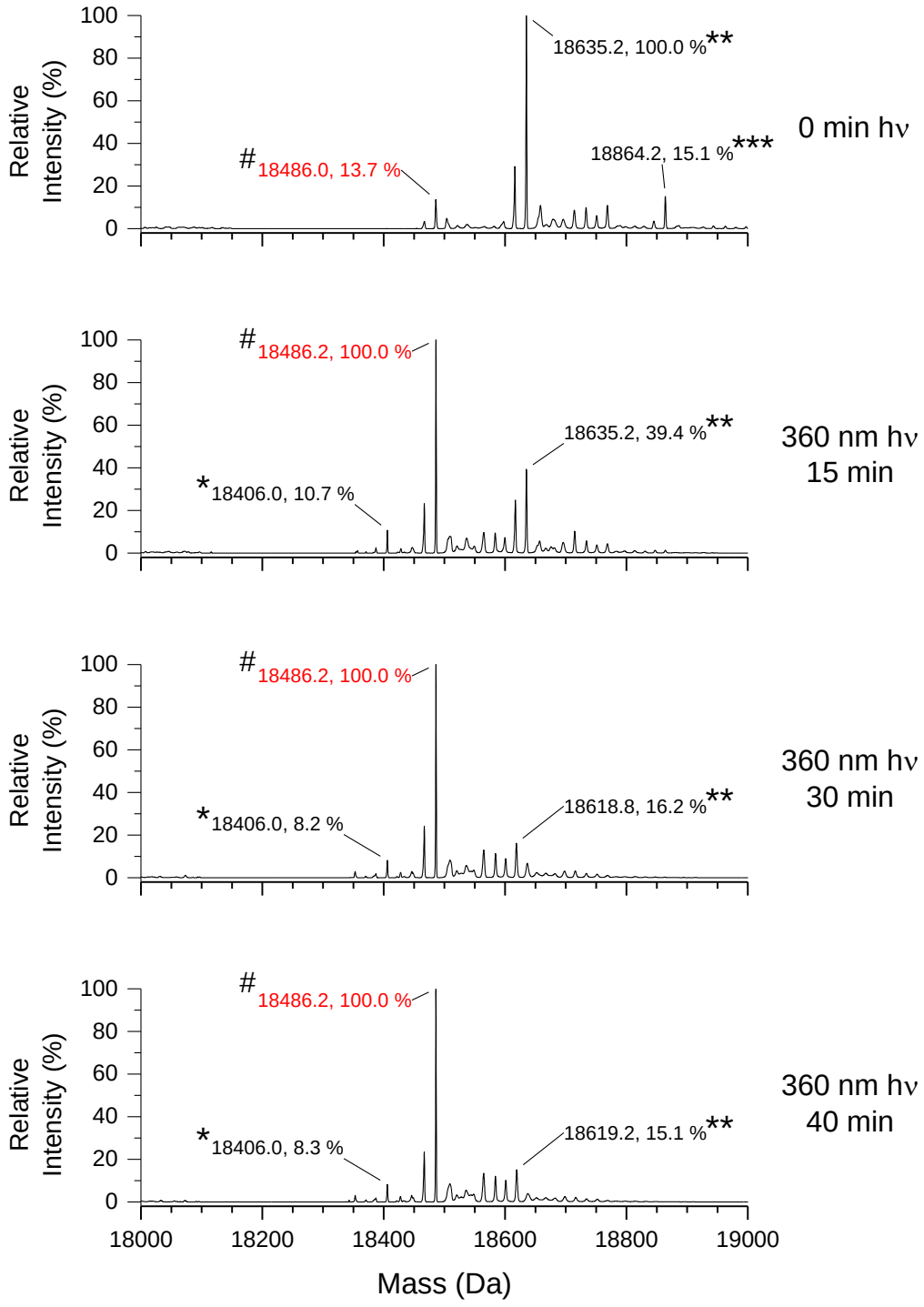
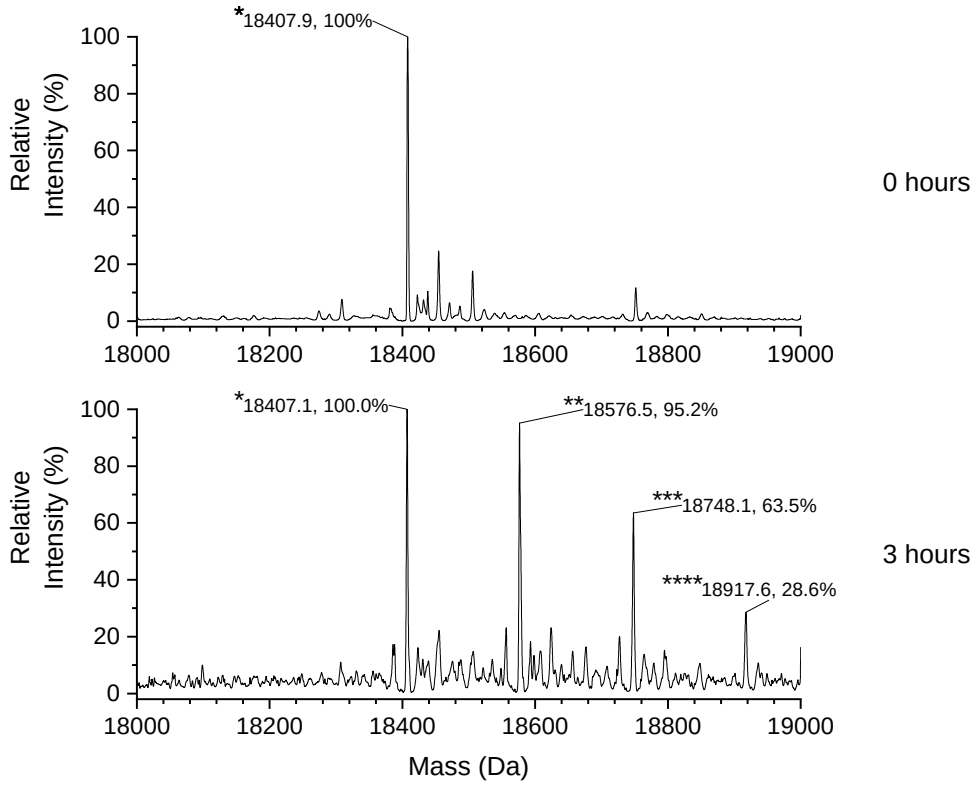
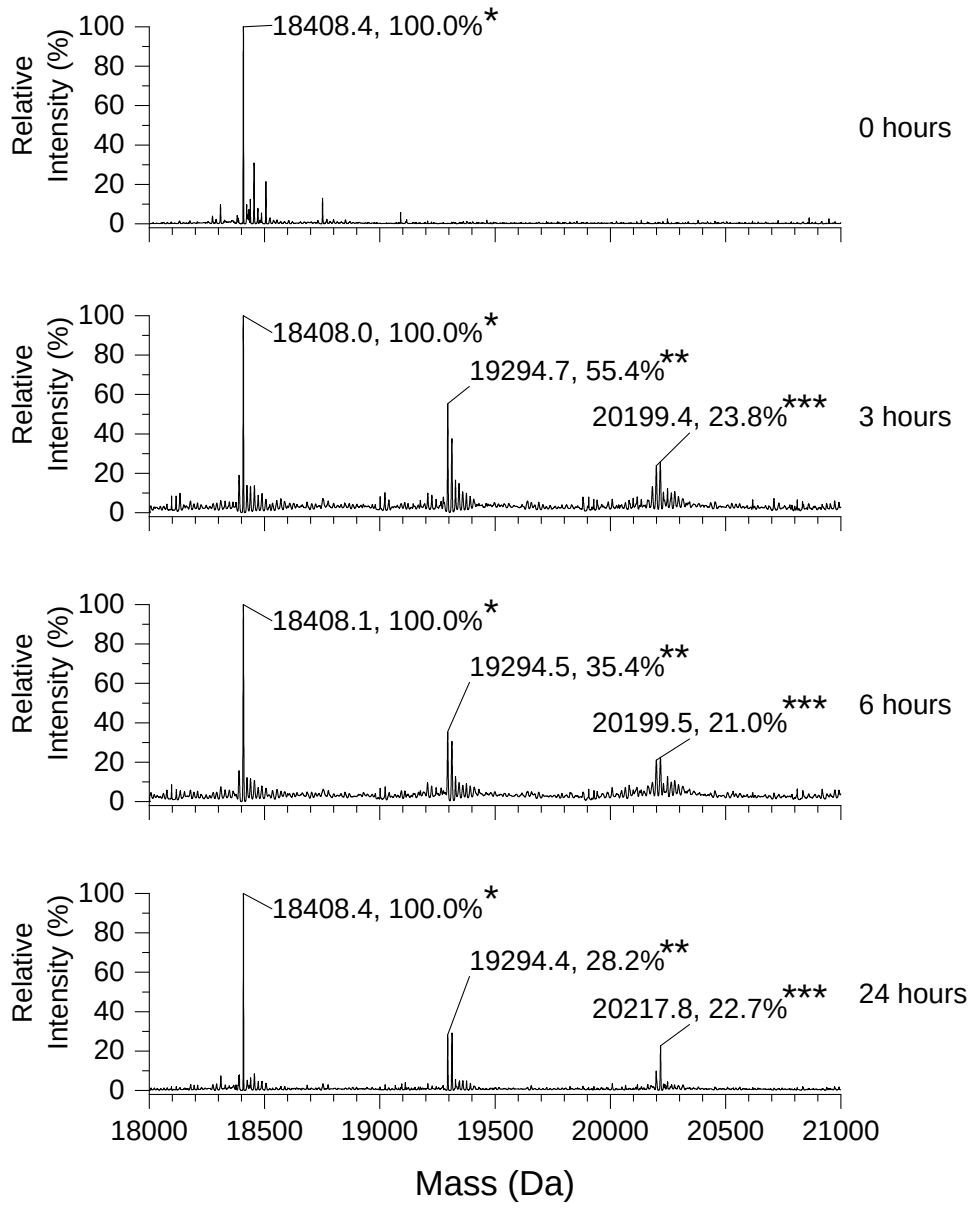


Figure S18. * = Myo-D127pS, ** = Myo-NPE-pp-S127, *** = Myo-D127pS +2x NPE-OPO_2, # = Myo-ppS127. ESI-MS timepoints of photo-irradiation of Myo-D127pS-OPO_2-NPE (synthesized by pyrophosphorylation of Myo-D127pS with reagent **2**) to yield Myo-D127ppS.



Supporting Figure S19. * = Myo-D127pS, ** = Myo-D127pS + (**1**-Im), *** = Myo-D127pS +2x(**1**-Im), **** = Myo-D127pS +3x(**1**-Im). ESI-MS timepoints of Myo-D127pS pyrophosphorylated with reagent **1** in aqueous solution without DMA.



Supporting Figure S20. * = Myo-D127pS, ** = Myo-D127pS + (**3**-Im), *** = Myo-D127pS +2x (**3**-Im), # = Myo-ppS127. ESI-MS timepoints of Myo-D127pS pyrophosphorylated with reagent **3** in aqueous solution without DMA.

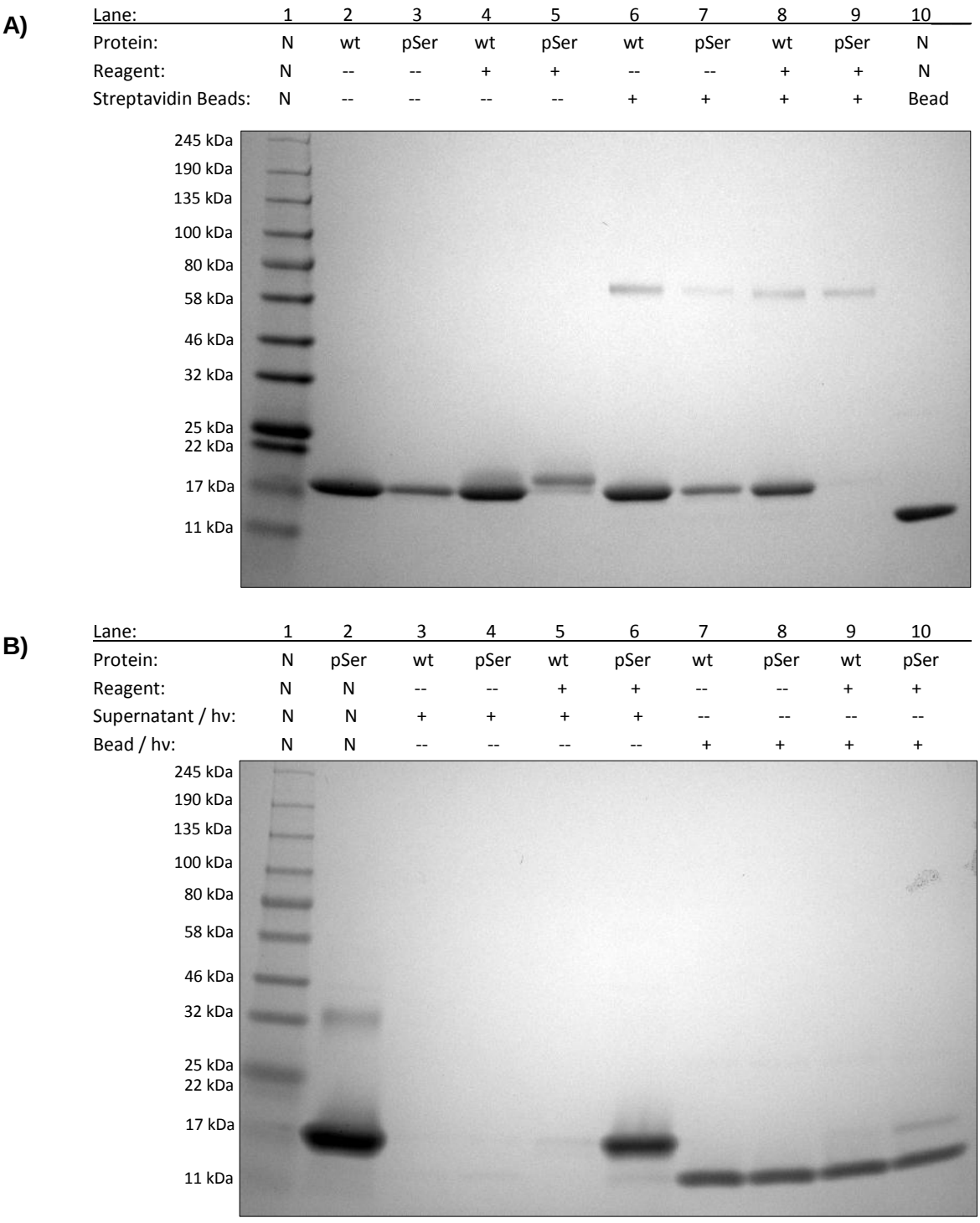


Figure S21. A) Full gel image corresponding to the segment shown in **Figure 4b**. Myoglobin aliquots after pyrophosphorylation with reagent **3** (lanes 2-5) and samples of the same protein aliquots after incubation with albumin-blocked streptavidin beads followed by concentration *via* spin filtration (lanes 6-9). Bands at ~70 kDa in lanes 6-9 are excess albumin from streptavidin bead blocking. Un-treated beads were loaded into lane 10. B) Full gel image corresponding to the

segment shown in **Figure 4c**. Streptavidin beads which had been exposed to myoglobin samples were re-suspended and irradiated with 360 nm light to photo-release any captured pyrophosphorylated protein. Supernatants of the streptavidin beads were concentrated *via* spin filtration and loaded into lanes 3 – 6. After washing, the aliquots of the beads themselves were loaded into lanes 7 – 10 to visualize protein that remains bound.

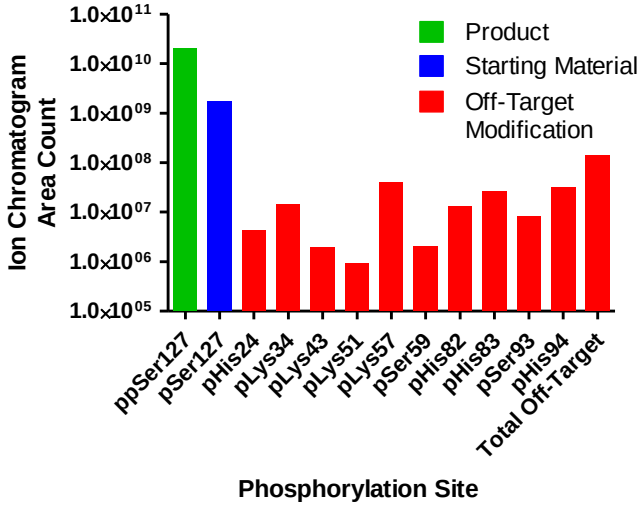


Figure S22. Ion abundances of phosphorylated peptides displayed on a log scale. Peptides are from tryptic digest of Myo-D127pS after pyrophosphorylation with reagent **2** and photo-deprotection.

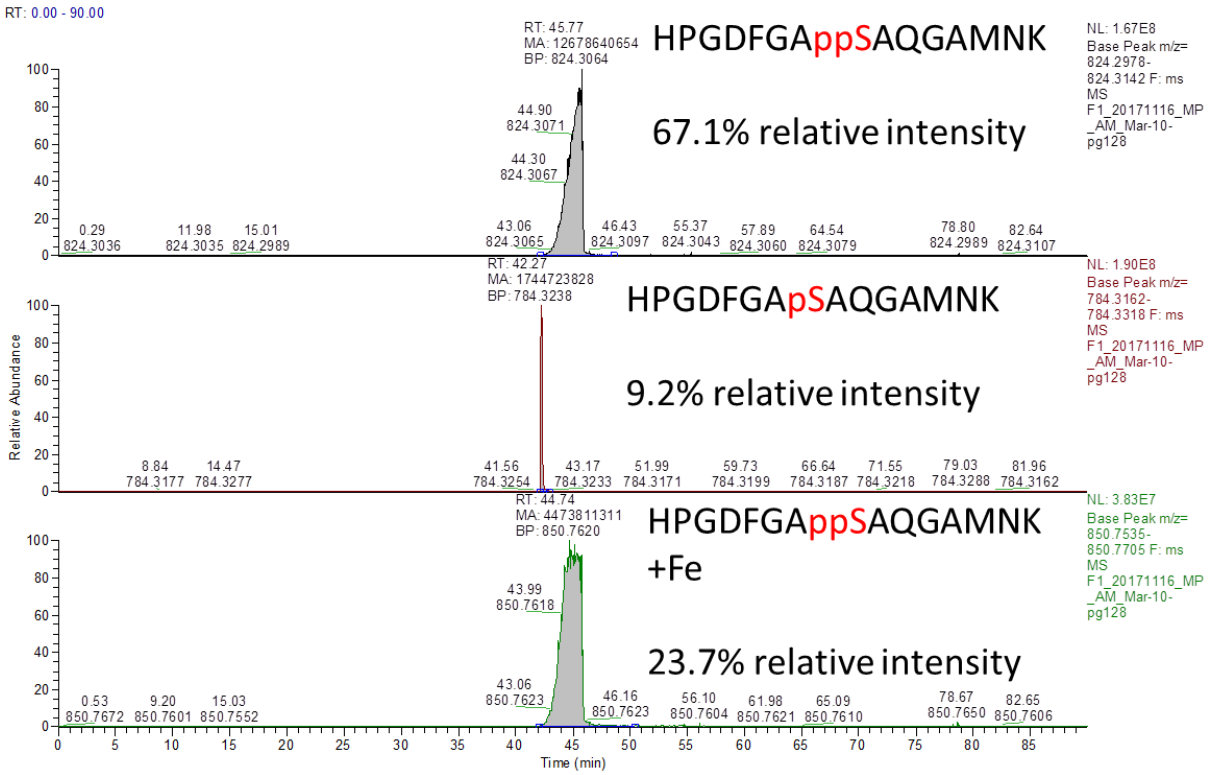


Figure S23. Single ion chromatograms of pyrophosphorylated peptides detected during bottom-up proteomics analysis of Myo-D127pS pyrophosphorylated with reagent **2** and subject to photo-deprotection.

II. Supporting Tables

Table S1. Reaction progress kinetics analysis of competition experiments.

Entry	Additive	Additive Conc. (mM)	Half-life (sec)	k_{obs} (sec^{-1})	k ($M^{-1}sec^{-1}$)
1	None	——	136.8	5.06×10^{-3}	0.185
2	$BnOPO_3H_2$ (**18**)	10.0	209.0	3.31×10^{-3}	ND
3	$BnOPO_3H_2$ (**18**)	60.0	358.4	1.89×10^{-3}	ND
4	Phenylacetic acid	60.0	149.8	4.62×10^{-3}	0.167
5	Citric acid	60.0	149.8	4.61×10^{-3}	0.166
6	Benzyl mercaptan	60.0	167.2	4.14×10^{-3}	0.148
7[a]	2-mercaptoethanol	60.0	188.7	3.42×10^{-3}	0.123
8[a]	Dithiothreitol	60.0	179.6	3.86×10^{-3}	0.136
9[a]	Guanidine HCl	60.0	854.8	7.92×10^{-4}	2.37×10^{-2}
10	Benzylamine HCl	60.0	1654.0	6.01×10^{-4}	1.67×10^{-2}
11	Benzylamine	60.0	NR	NR	NR

Reaction conditions: 30 mM phosphorimidazolide **1**, 10 mM phosphopeptide **14**, 80 mM $ZnCl_2$, in 1:9 H_2O:DMA at 45 °C. Values are calculated using time-points which are averages from three replicate reactions monitored from 0 to 1020 sec. Calculations are given in the kinetics section. Note: a) Data from a single reaction was used instead of three replicates.

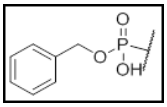
Table S2. List of phosphorylated peptides detected by EThcD and their ion counts.

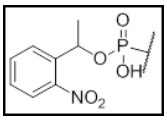
Phosphorylated Residue	Sequence	Ion Chromatogram Area Counts	% of Total
pHis24	VEADVAGpHGQDILIR	4232319	0.02%
pLys34	LpKSHPETLEK	14083066	0.06%
pLys43	SHPETLEpKFDR	1980651	0.01%
pLys51	FKHLpKTEAEMK	941832	0.00%
pLys57	TEAEMpKASEDLKK	40494925	0.18%
pSer59	TEAEMKApSEDLKK	2080558	0.01%
pHis82	KGpHHEAELKPLAQSHATK	12773803	0.06%
pHis83	KGHpHEAELKPLAQSHATK	26432138	0.12%
pSer93	GHHEAELKPLAQpSHATK	8199602	0.04%
pHis94	GHHEAELKPLAQSpHATK	31649728	0.14%
ppSer127	HPGDFGAppSAQGAMNK	12706307522	57.40%
ppSer127	HPGDFGAppSAQGAoxidMNK	3151983559	14.24%
ppSer127	HPGDFGAppSAQGAMNK+Fe	4426375152	20.00%
pSer127	HPGDFGApSAQGAMNK	1709518746	7.72%
total ppSer127	ppS127	20284666233	91.63%
total off-target	pH 24, 82, 83, 94; pK 43, 51, 57; pS 59, 93	142868622	0.65%

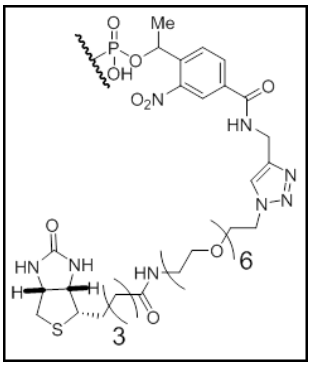
Bottom-up proteomics analysis of Myo-ppSer127 synthesized using photo-labile phosphorimidazolide reagent **2**.

Table S3. Calculated masses of proteins, phosphorimidazolide reagent adducts, and free pyrophosphates.

Proteins	MW (Daltons)							
Name	Un-modified Protein	1x (**1**-Im)	2x (**1**-Im)	1x (**2**-Im)	2x (**2**-Im)	1x (**3**-Im)	2x (**3**-Im)	Free Pyrophosphate
Ub-wt	8564.816	8734.92	8905.02	8793.94	9023.07	9451.73	10338.64	
Ub-S65pS	8644.794	8814.90	8985.00	8873.92	9103.05	9531.71	10418.62	8724.772
Myo-wt	18355.07	18525.18	18695.28	18584.20	18813.33	19241.98	20128.90	
Myo-D127pS	18407.04	18577.14	18747.25	18636.17	18865.30	19293.95	20180.86	18487.02
Myo-G154C	18401.16	18571.26	18741.37	18630.29	18859.42	19288.07	20174.98	
Myo-D127pS-G154C	18453.13	18623.23	18793.34	18682.26	18911.39	19340.04	20226.95	18533.11
		(+170.104)	(+340.208)	(+229.128)	(+458.256)	(+886.912)	(+1773.824)	(+79.978)

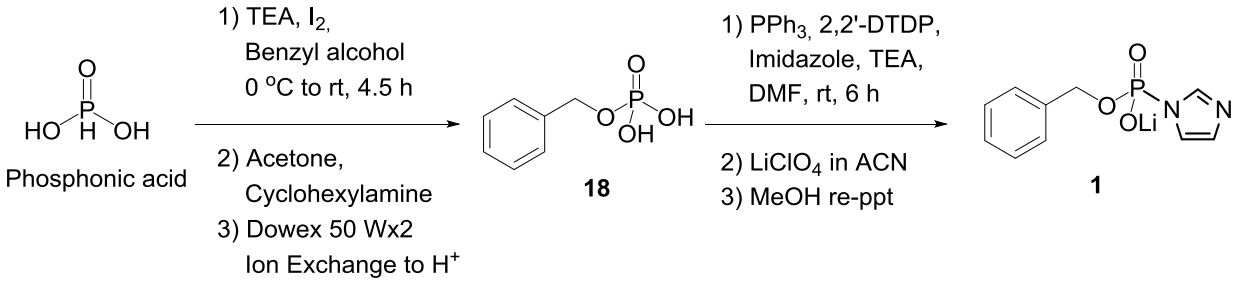




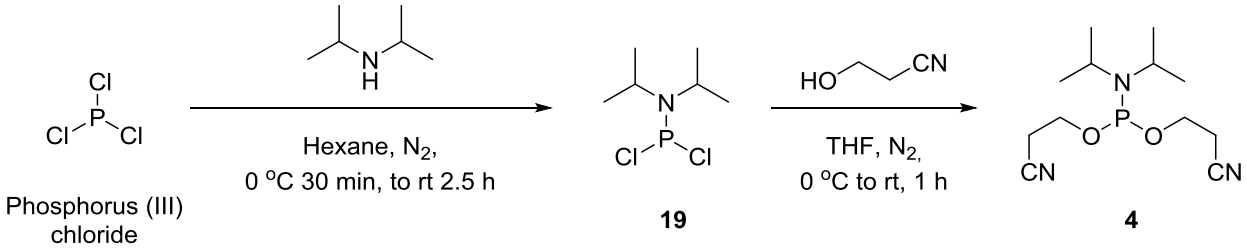


III. Supporting Schemes

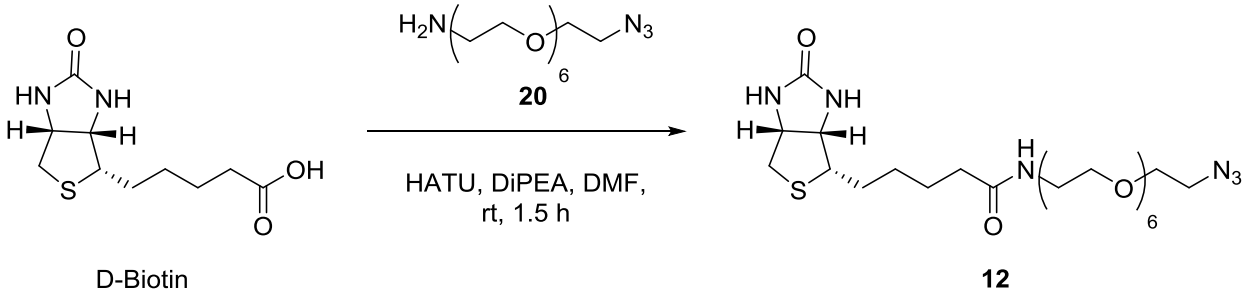
Scheme S1. Synthesis of Phosphorimidazolide Reagent (1)



Scheme S2. Synthesis of phosphoramidite reagent (4)



Scheme S3. Synthesis of Biotin-PEG6-Et-N3 (17)



IV. Chemical Synthesis and Characterization

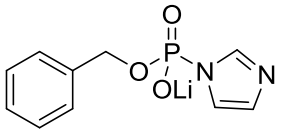
General Information and Instrumentation:

Commercially available chemicals were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics, Strem Chemicals, TCI America, Anaspec, Carl Roth Gmbh., and Iris. Dichloromethane and THF were dried by passing through an activated alumina column, and acetonitrile, DMSO, and DMF were dried by passing through a column of activated molecular sieves using a Pure Process Technology drying system. All other reagents were used as received unless otherwise specified. Thin layer chromatography (TLC) was conducted on EMD Silica Gel 60 F254 plates and visualized by fluorescence quenching. Automated column chromatography was conducted using SiliCycle SiliaFlash F60 (40-53 μm) silica dry-loaded in RediSep® Rf cartridges and normal-phase silica flash columns on a CombiFlash® Rf system from Teledyne Isco.

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker 500 AVANCE spectrometer (500, 125, and 203 MHz respectively) with cryocool. NMR data are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, dt = doublet of triplets, dq = doublet of quartets, dp = doublet of pentets, m = multiplet, b = broad peak), coupling constant (Hz), and integration. ^{31}P NMR spectra were referenced to a 500 mM tetramethylphosphonium bromide ($P(CH_3)_4Br$) standard (22.77 ppm) in a D_2O capillary insert. Solutions of deuterium chloride or sodium deuteroxide in D_2O were used to adjust the pD for NMR spectra of compounds in D_2O. pD values were determined by applying the following correction[3] to the measured pH value (pH*): pD = 0.929pH* + 0.42.

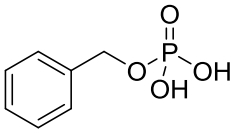
High resolution mass spectra (HRMS) were acquired on an Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF) spectrometer at the Princeton University Department of Chemistry. For positive ion mode detection, 3% H_2O in ACN with 0.1% formic acid was used, and for negative ion mode detection, 50% H_2O in ACN with 2.5 mM ammonium acetate was used. HRMS of reagent **3**, and its synthetic precursors (**7**, **8**, **9**, **10**, **11**, **12**, and **13**) were acquired on an Agilent Technologies 6230 Accurate Mass TOF LC/MS linked to Agilent Technologies 1260 Series HPLC at the Leibniz Research Institute for Molecular Pharmacology. Column: Thermo Accucore RP-MS (2.6 μM, 30 x 2.1 mm); Eluent A: H_2O with 0.1 % Formic Acid Eluent B: ACN with 0.1 % Formic Acid; Conditions: 0.00 min 95 % A, 0.2 min 95% A, 2.1 min to 1% A as gradient, 3 min as Stop time, 1.5 min Post time for reconstitution; Flow rate: 0.8 ml/min; UV-detection: 220 nm, 254 nm, 450 nm.

Low resolution mass spectra (LRMS) were acquired on an Agilent Technologies 6130 Quadrupole LCMS with an Agilent 1260 series solvent pump, auto sampler, column oven, and diode array detector (monitoring UV 254 and 280 nm). Compounds were separated with a gradient of 10% to 90% ACN in a solution of H_2O with 0.1% FA over 3.4 min with a flow rate of 0.7 mL/min. An Agilent SB C18 column was used (1.8 μm, 2.1 x 50 mm). Instrument control and data processing was conducted with Open Lab CDS software (Chem Station Edition for LC & LC/MS systems © 2015).



Lithium benzyl 1H-imidazol-1-ylphosphonate (1)

A solid mixture of monobenzylphosphate (**18**) (3.1533 g, 16.7631 mmol), 2,2'-dithiodipyridine (11.0799 g, 50.2892 mmol), Imidazole (11.4126 g, 167.6306 mmol), and triphenylphosphine (13.1893 g, 50.2892 mmol) were dissolved in 81 mL of DMF before adding 7.0 mL (50.4 mmol) of neat TEA. The resulting clear, yellow solution was allowed to stir at r.t. for 6 hours before diluting with a solution of $LiClO_4$ (7.1338 g, 67.1 mmol) in 271 mL of ACN. A white ppt formed upon dilution. The ppt was collected *via* vacuum filtration, rinsing forward and washing with ACN to remove yellow color. To remove the remaining yellow impurity, the ppt was re-dissolved in the minimum volume of methanol and passed through a 0.45 μm PTFE whatman filter. The resulting clear solution was then diluted with ACN until the ppt re-formed. The ppt was again collected by vacuum filtration, washed with ACN, air dried, then ground to a powder and further dried *in vacuo*. The title compound was obtained as 2.971 g (73 %) of a white solid. ^{1}H NMR (600 MHz, D_2O, pD = 8.83): δ 7.83 (s, 1H), 7.36 – 7.35 (m, 3H), 7.26 – 7.25 (m, 2H), 7.22 (s, 1H), 7.03 (s, 1H), 4.89 – 4.88 (d, *J* = 9.31 Hz, 2H); ^{13}C NMR (151 MHz, D_2O, pD = 8.83): δ 142.6, 138.8 (d, *J* = 5.71 Hz), 131.6, 131.4, 131.2, 130.5, 122.9 (d, *J* = 4.14 Hz), 71.3 (d, *J* = 5.10 Hz); ^{31}P NMR (243 MHz, D_2O, pD = 8.83): δ -8.15 (t, *J* = 8.18 Hz). HRMS (*m/z*): $[M-Li+2H]^+$ calcd for $C_{10}H_{12}N_2O_3P^+$ 239.05801; found, 239.0595.

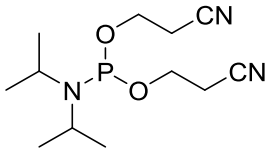


Monobenzylphosphate (18)

Monobenzylphosphate was prepared using a procedure adapted from Kahn et al.[4]. 3.339 g (40.718 mmol) of solid phosphonic acid stored over P_2O_5 was dissolved in 67.9 mL of neat benzyl alcohol and 19.9 mL of neat TEA (142.5 mmol). The resulting clear, colorless solution was cooled in an ice bath. 15.50 g of solid I_2 (61.08 mmol) was slowly added in portions over 5 min with stirring. After stirring at 0 ˚C for 10 min the solution was allowed to warm to room temperature and continue stirring for a further 4.5 hours*. In a separate flask, 14.0 mL (122.2 mmol) of cyclohexylamine (CHA) was dissolved in 263 mL of acetone. The black-orange reaction mixture was diluted into the solution of CHA in acetone and a voluminous white ppt immediately formed.

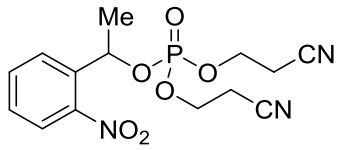
* Note that significantly longer reaction times may result in side-product formation while significantly shorter reaction times may result in incomplete consumption of phosphonic acid.

The ppt was collected *via* vacuum filtration, rinsing forward and washing with acetone. The ppt was then recrystallized from a solution of 15:85 H_2O:EtOH with 1% CHA. The resulting white, needle-like crystals were collected *via* vacuum filtration, then washed with ice-cold 15% H_2O in ethanol with 1% CHA, followed by acetone. Four additional crops were recrystallized and collected from product that re-precipitated during the final acetone wash. At this stage, purity of the recrystallized material should be checked by ^{31}P NMR to ensure that product is free of phosphonic acid starting material which is not rejected by recrystallization. Pure recrystallized product is dissolved in D.I. water and ion exchanged to the free acid by passing through a Dowex 50 Wx2 100-200 mesh cation exchange column (0.6 mmol/mL wet bed volume) in H^+ form. Eluent was concentrated to small volume (~60 mL) *via* rotory evaporation, distributed into tared glass vials, frozen in liquid N_2, and lyophilized to give 4.8013 g (62.7%) of the title compound as a white solid. ^1H NMR (300 MHz, D_2O, pD = 7.81): δ 7.30 – 7.14 (m, 5H), 4.63 (d, *J* = 5.26 Hz, 2H); ^{13}C NMR (75 MHz, D_2O, pD = 7.81): δ 138.8 (d, *J* = 8.34 Hz), 128.4, 127.5, 127.4, 65.8 (d, *J* = 4.33 Hz); ^{31}P NMR (122 MHz, D_2O, pD = 7.81): δ 3.69 (t, *J* = 5.25 Hz). HRMS (*m/z*): $[M-H]^-$ calcd for $C_7H_8O_4P^-$ 187.01657; found, 187.0173. $[2M-H]^-$ calcd for $C_{14}H_{17}O_8P^-$ 375.04041; found, 375.0412.



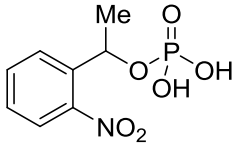
N,N-diisopropyldicyanoethylphosphoramidite (4)

The following protocol is based on a procedure from Uhlmann *et al*[5]. To a dry flask equipped with stir-bar under N_2 was added 35 mL of dry THF. The apparatus was cooled in an ice bath. Neat DiPEA (21.22 g, 164.2 mmol) and neat 3-hydroxypropionitrile (8.21 g, 116 mmol) were added with stirring at 0 ˚C. Neat 1,1-dichloro-N,N-diisopropylphosphanamine (**19**) was added dropwise over 10 min and rinsed forward with 3 mL of dry THF. A white ppt formed during the addition of **19** and the resulting suspension was allowed to warm to rt and stir vigorously for 1 h. The suspension was filtered through a porous frit and rinsed forward extensively with THF and hexanes. The filtrate was concentrated *via* rotory evaporation to yield a cloudy green oil. The crude material was subject to f.c. isocratically with 80:15:5 hexanes:EtOAc:TEA. Product-containing fractions were combined, concentrated *via* rotory evaporation, and dried *in vacuo*. The title compound was obtained as 10.0310 g (60.8 %) of a viscous, clear, pale brown-yellow liquid. ^1H NMR (300 MHz, D_3-ACN): δ 3.89 – 3.72 (m, 4H), 3.69 – 3.54 (m, 2H), 2.66 (t, *J* = 5.97 Hz, 4H), 1.18 (d, *J* = 6.81 Hz, 12H). ^{13}C NMR (75.6 MHz, D_3-ACN): δ 118.4, 58.5 (d, *J* = 19.21 Hz), 42.9 (d, *J* = 12.34 Hz), 23.8 (d, *J* = 7.30 Hz), 19.9 (d, *J* = 6.99 Hz). ^{31}P NMR (121.5 MHz, D_3-ACN): δ 148.0 (t, *J* = 7.66 Hz). HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{12}H_{22}N_3NaO_2P^+$ 294.1342; found, 294.1344.



1-(2-nitrophenyl)ethyl-*O*-biscyanoethyl phosphate (5)

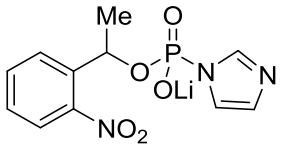
1-(2-nitrophenyl)ethyl phosphate **5** was prepared according to a procedure adapted from Corrie et al.[6] A sample of dry 2-nitroephnylethanol (1.020 g, 6.102 mmol) was dissolved in dry ACN. Neat bis(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (**4**) (2.069g, 7.627 mmol) was added with stirring followed by 1*H*-tetrazole as a 0.45 M solution in ACN (20.3 mL, 9.14 mmol). The resulting clear, light yellow solution was allowed to stir at rt for 3 hours. While stirring, a white ppt formed and the solution became cloudy. The solution was cooled to -40 ˚C in a dry ice / ACN bath and *t*-butylhydroperoxide as a 5.5 M solution in decane (1.85 mL, 10.16 mmol) was added dropwise over one minuet. After stirring at -40 ˚C for 20 minutes, the solution was allowed to warm to rt and stir for a further 70 minutes. 40 mL of aqueous 5% $Na_2S_2O_3$ was added. After 10 minutes of vigorous stirring, the reaction mixture was diluted with 160 mL of EtOAc. The aqueous layer was drained and the organic layer was washed with D.I. H_2O, dried over $MgSO_4$, filtered, concentrated, and dried *in vacuo* to give a yellow liquid. The crude product was partially purified *via* f.c. with 0 – 10% MeOH in Et_2O to give 1.1909 g (approx. 3.37 mmol, 43%) of the title compound as a yellow liquid. The material was taken forward to the next reaction after partial characterization. ^{1}H NMR (300 MHz, d_3-ACN) δ 8.00 (d, *J* = 8.21 Hz, 1H), 7.85, (dd, *J*1 = 1.07 Hz, *J*2 = 7.82 Hz, 1H), 7.78 (t, *J* = 7.64, 1H), 7.57 (dt, *J*1= 1.43 Hz, *J*2 = 7.74 Hz, 1H), 6.05 (p, *J* = 6.58 Hz, 1H), 4.28 – 4.04 (m, 4H), 2.76 (t, *J* = 5.92 Hz, 2H), 2.69 (t, *J* = 5.92 Hz, 2H), 1.72 (d, *J* = 6.34 Hz, 3H). ^{31}P NMR (203 MHz, D_2O) -3.78 (dp, *J*1 = 7.78 Hz, *J*2 = 7.81 Hz).



1-(2-nitrophenyl)ethyl phosphate (6)

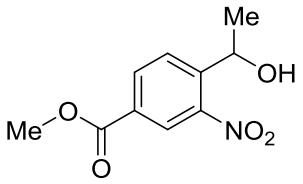
6 was prepared according to a procedure adapted from Correi et al.[6]. Phosphate triester **5** (1.060 g, 3.001 mmol) was dissolved in 75 mL of 0.1M NaOH in methanol. The resulting clear, red solution was heated to 50 ˚C, and stirred for 30 min, during which time it slowly turned yellow. The solution was concentrated *via* rotory evaporation to give an oily, brown-white residue. The residue was suspended in 20 mL of D.I. water, passed through a 0.45 μm nylon whatman filter, and rinsed forward with 5 mL of D.I. water to yield a clear, brown solution. 1.50 mL of aqueous 2 M $Ba(OAc)_2$ was added and the solution developed a white haze. The solution was then diluted with Absolute EtOH until a white ppt formed and persisted (150 mL). The ppt was collected *via* vacuum

filtration and the wet filter cake was rinsed with ice-cold 1:1 H_2O:EtOH, followed by Et_2O, then aspirated for 10 min. The ppt was ground into fine chunks then dried *in vacuo* to yield 855 mg of a pale white-brown solid. This barium salt was re-suspended in water and stirred with the assistance of sonication until all chunks had been broken apart, then passed through a Dowex 50 Wx2 100-200 mesh resin cation exchange column (0.6 mmol/mL wet bed volume) in H^+ form with 500 mL of D.I. water. The eluent was concentrated *via* rotory evaporation then dried *in vacuo* to give 0.5335 g (2.159 mmol, 72%) of the title compound as a yellow liquid. ^{1}H NMR (501 MHz, D_2O, pD = 7.8) δ 7.93 (d, *J* = 8.21 Hz, 1H), 7.84 (d, *J* = 7.80 Hz, 1H), 7.66 (t, *J* = 7.45 Hz, 1H), 7.38 (t, *J* = 7.90 Hz, 1H), 5.68 (dq, *J*1 = 6.37 Hz, *J*2 = 8.48 Hz, 1H), 1.43 (d, *J* = 6.31 Hz, 3H); ^{13}C NMR (126 MHz, D_2O, pD = 7.8) δ 146.3, 141.3 (d, *J* = 3.18 Hz), 134.2, 128.0, 127.7, 124.3, 68.1 (d, *J* = 3.94 Hz), 24.2 (d, *J* = 5.04 Hz); ^{31}P NMR (203 MHz, D_2O, pD = 7.8) δ 3.01 (d, *J* = 8.45 Hz). HRMS (*m/z*): $[M-H]^-$ calcd for $C_8H_9NO_6P^-$ 246.0173; found, 246.01704; $[2M-H]^-$ calcd for $C_{16}H_{19}N_2O_{12}P_2^-$ 493.04187; found, 493.04175.



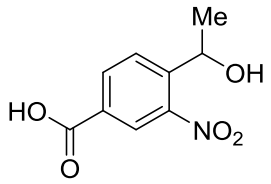
Lithium 1-(2-nitrophenyl)ethyl 1H-imidazol-1-ylphosphonate (2)

A mixture of **6**, (1.7262 g, 6.9847 mmol), 2,2'-dithiodipyridine (4.617 g, 20.954 mmol), imidazole (4.755 g, 69.847 mmol), and triphenylphosphine (5.496 g, 20.954 mmol) were dissolved in 33.7 mL of DMF before adding 2.92 mL (20.95 mmol) of neat TEA. The resulting clear, yellow solution was allowed to stir at rt for 6 hours before diluting with a solution of 2.972 g (27.939 mmol) $LiClO_4$ in 17.0 mL of ACN. More ACN antisolvent was added until a white ppt formed. The ppt was collected *via* vacuum filtration and the wet filter cake was washed with ACN. The ppt was re-dissolved in the minimum amount of MeOH (~7 mL) then ACN was added until the ppt re-formed. The ppt was again collected *via* vacuum filtration, ground, then dried *in vacuo* to yield 1.2582 g (4.1506 mmol, 59.4 %) of the title compound as a granular white-brown solid. ^{1}H NMR (600 MHz, D_2O, pD = 8.06) δ 7.75 (dd, *J*1 = 0.97 Hz, *J*2 = 8.20 Hz, 1H), 7.55 (dd, *J*1 = 6.30 Hz, *J*2 = 7.93 Hz, 1H), 7.52 (dt, *J*1 = 0.89 Hz, *J*2 = 7.04 Hz, 1H), 7.47 (s, 1H), 7.32 (dt, *J*1 = 1.70 Hz, *J*2 = 7.69 Hz, 1H), 6.87 (q, *J* = 1.38 Hz, 1H), 6.67 (s, 1H), 5.66 (dq, *J*1 = 6.39 Hz, *J*2 = 8.88 Hz, 1H), 1.48 (d, *J* = 6.40 Hz, 3H); ^{13}C NMR (151 MHz, D_2O, pD = 8.06) δ 146.2, 139.3 (d, *J* = 5.48 Hz), 137.0, 134.3, 128.7, 127.7, 124.2, 119.8 (d, *J* = 5.89 Hz), 70.9 (d, *J* = 5.15 Hz), 23.5 (d, *J* = 6.92 Hz); ^{31}P NMR (122 MHz, D_2O, pD = 8.06) δ -9.57 (d, *J* = 8.75 Hz). HRMS (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{12}LiN_3O_5P^+$ 304.06691; found, 304.06505; $[M-Li+2H]^+$ calcd for $C_{11}H_{13}N_3O_5P^+$ 298.05873; found, 298.05686.



Methyl 4-(1-hydroxyethyl)-3-nitrobenzoate (7)

Alcohol **7** was synthesized using a protocol adapted from Imwinkelried et al.[7]. To an oven-dried schlenk flask equipped with stir-bar under N_2 was added 2.79 mL of neat $Ti(OiPr)_4$ (2.67 g, 9.41 mmol). After cooling to 0 ˚C in an ice bath, 0.344 mL (0.595 g, 3.136 mmol) of neat $TiCl_4$ was added dropwise. The liquids solidified. 11.4 mL of dry THF was added and the mixture was allowed to warm to rt. After the solids dissolved, the clear, colorless solution was allowed to stir at rt for a further 30 min. The solution was again cooled to 0 ˚C then 7.84 mL of 1.6 M MeLi in hexanes[†] (12.54 mmol) was added dropwise over 9 min. The solution immediately turned bright yellow. The resulting clear, yellow solution was allowed to stir at 0 ˚C for a further 1h. In a separate dry flask, 2.234 g (12.543 mmol) of Methyl 4-formyl-3-nitrobenzoate was dissolved in 10.8 mL of dry THF and then added dropwise to the methyl titanium reagent solution over 15 min while maintaining the reaction at 0 ˚C. The reaction mixture darkened and was allowed to stir at 0 ˚C for an additional 70 min before quenching into ice-cold aqueous 1M HCl. The aqueous layer was separated and extracted with EtOAc (3x equal volumes). All organic fractions were combined, rinsed with D.I. H_2O (1x), sat. $NaHCO_3$ (1x), and brine (1x), then dried over Na_2SO_4, filtered, and concentrated onto silica gel *via* rotory evaporation. The crude product was purified *via* f.c. with a gradient of 1-5% MeOH in DCM. 2.444 g (86.5%) of purified product was obtained as a yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, J = 1.34 Hz, 1H), 8.29 – 8.25 (dd, $J1$ = 1.29 Hz, $J2$ = 8.21 Hz, 1H), 7.97 – 7.94 (d, J = 8.21 Hz, 1H), 5.51 – 5.45 (q, J = 6.33 Hz, 1H), 3.96 (s, 3H), 2.35 (s, 1H, broad), 1.59 – 1.57 (d, J = 6.37 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.8, 147.5, 145.5, 134.0, 130.3, 128.0, 125.5, 65.6, 52.7, 24.4. HRMS

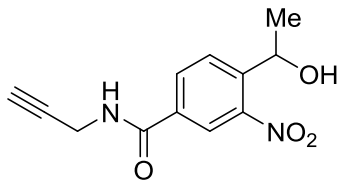


4-(1-hydroxyethyl)-3-nitrobenzoic acid (8)

To a round-bottom flask was added 2.2067 g (1.2345 mmol) of Methyl 4-(1-hydroxyethyl)-3-nitrobenzoate (**7**) dissolved in 233 mL of ACN followed by 233 mL of 0.5 M aqueous NaOH. The

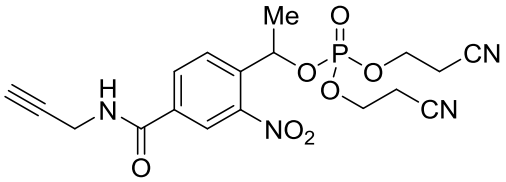
[†] Recently titrated with diphenylacetic acid

resulting biphasic mixture was heated to 50 ˚C and stirred vigorously for 20 min. The mixture was allowed to cool to rt before adding 100 mL of Et_2O and separating the aqueous layer. The aqueous layer was adjusted to acidic pH using 6 M HCl and then extracted with Et_2O (3x). The yellow organic extracts were combined, washed with brine (1x), dried over Na_2SO_4, filtered, and concentrated *via* rotory evaporation. The crude product was purified *via* f.c. with a gradient of 0-70 % EtOAc with 0.2 % F.A. in hexanes. 1.619 g (78.2%) of sticky yellow solid was obtained. ^{1}H NMR (300 MHz, CD_3-CN) δ 8.40 (d, *J* = 1.37 Hz, 1H), 8.27 – 8.24 (dd, *J*1 = 1.35 Hz, *J*2 = 8.20 Hz, 1H), 7.98 – 7.95 (d, *J* = 8.21 Hz, 1H), 5.36 – 5.30 (q, *J* = 6.38 Hz, 1H) 1.49 – 1.47 (d, *J* = 6.38 Hz, 3H); ^{13}C NMR (75 MHz, CD_3-CN) δ 164.9, 147.6, 146.3, 133.6, 130.1, 128.2, 124.9, 64.7, 23.9. HRMS $[M+H]^+$ calcd for $C_9H_{10}NO_5^+$ 212.0554, found 212.0548.



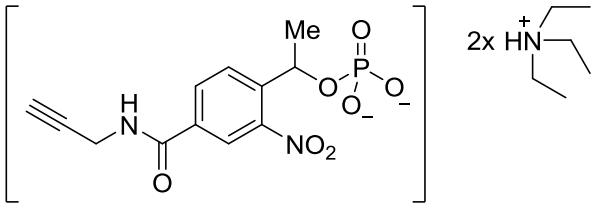
4-(1-hydroxyethyl)-3-nitro-N-(prop-2-yn-1-yl)benzamide (9)

1.6190 g (7.667 mmol) of solid 4-(1-hydroxyethyl)-3-nitrobenzoic acid (**8**) and 3.2067 g (8.433 mmol) of solid HATU were dissolved in 16.9 mL of DMF. 2.79 mL (16.87 mmol) of neat DiPEA was added with stirring to give a clear, red solution, followed immediately by 1.964 mL (1.689 g, 30.667 mmol) of neat propargylamine (clear, bright yellow). The resulting clear, red-yellow solution was allowed to stir at rt for 3h, then diluted with 50 mL of EtOAc. The solution was washed with D.I. H_2O (3x) until the aqueous fractions became colorless. The organic layer was further washed with brine (1x), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude material was purified *via* f.c. with a gradient of 1.5 – 4.5% MeOH in DCM. 1.327 g (70.0 %) of red-brown oil was obtained with several impurities. Partial characterization was performed and product was carried on to the next step without further purification. ^{1}HNMR (300.2 MHz, d_3-ACN) δ 8.28 (d, *J* = 1.69 Hz, 1H), 8.08 (dd, *J*1 = 1.68 Hz, *J*2 = 8.22 Hz, 1H), 7.94 (d, *J* = 8.21 Hz, 1H), 7.59 (bs, 1H), 5.33 (dq, *J*1 = 4.18 Hz, *J*2 = 6.31 Hz, 1H), 4.16 (dd, *J*1 = 2.52 Hz, *J*2 = 5.61 Hz, 2H), 2.51 (t, *J* = 2.50 Hz, 1H), 1.48 (d, *J* = 6.37 Hz, 3H). LRMS (*m/z*): $[M+H]^+$ calcd for $C_{12}H_{13}N_2O_4^+$ 249.09; found, 249.1. $[2M+H]^+$ calcd for $C_{24}H_{25}N_4O_8^+$ 497.17; found, 497.2.



Bis(2-cyanoethyl) (1-(2-nitro-4-(prop-2-yn-1-ylcarbamoyl)phenyl)ethyl) phosphate (10)

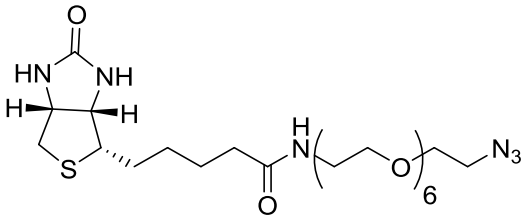
1.1687 g (4.708 mmol) of alcohol **9** were dissolved in 72.4 mL of dry ACN. To the resulting clear, yellow solution was added 1.660 g (6.120 mmol) of neat bis(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (**4**) followed by 27.5 mL (12.2 mmol) of 1*H*-tetrazole as a 0.45 M solution in ACN. After 10 min, the solution became cloudy and was allowed to continue stirring at rt overnight. The reaction mixture was then cooled to 0 ˚C in an ice bath and 1.43 mL (7.84 mmol) of *t*-butylhydroperoxide as a 5.5 M solution in decane was added dropwise. The solution was allowed to warm to rt and stir for 1h before the reaction was quenched into 5% aqueous NaS_2O_3. 100 mL of EtOAc was added and the aqueous layer was removed. The organic layer was washed with D.I. H_2O (2x), and brine (1x), dried over Na_2SO_4, then concentrated *via* rotory evaporation. The crude material was purified *via* f.c. with a gradient of 2 – 5% MeOH in DCM. 0.9243 g (45.2%) of the title compound was obtained as a yellow liquid. ^{1}H NMR (300 MHz, CD_3-CN) δ 8.42 (d, *J* = 1.52 Hz, 1H), 8.18 – 8.15 Hz (dd, *J*1 = 1.49 Hz, *J*2 = 8.23 Hz, 1H), 7.96 – 7.94 (d, *J* = 8.24 Hz, 1H), 7.70 (s, 1H), 6.11 (pentet, *J* = 6.47 Hz, 1H), 4.30 – 4.10 (m, 6H), 2.79 (t, *J* = 5.89 Hz, 2H), 2.72 (t, *J* = 5.96 Hz, 2H), 2.52 (t, *J* = 2.46 Hz, 1H), 1.75 – 1.73 (d, *J* = 6.36 Hz, 3H); ^{13}C NMR (75 MHz, CD_3-CN) δ 164.0, 146.8, 139.7 (d, *J* = 4.81 Hz), 134.8, 132.3, 128.3, 123.5, 79.8, 72.7, 72.6, 71.1, 62.7 (t, *J* = 5.41 Hz), 28.8, 23.1 (d, *J* = 5.51 Hz), 19.1 (dd, *J*1 = 3.23 Hz, *J*2 = 7.72 Hz); ^{31}P NMR (122 MHz, CD_3-CN) δ -3.64 (sextet, *J* = 7.32 Hz). HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{16}H_{20}N_3NaO_8P^+$ 436.08802, found 436.0882.



1-(2-nitro-4-(prop-2-yn-1-ylcarbamoyl)phenyl)ethyl dihydrogen phosphate (11)

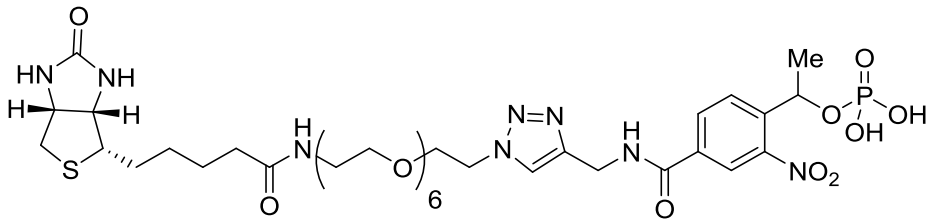
To a round bottom flask was added 0.924 g (2.13 mmol) of phosphate triester **10** dissolved in 55 mL of 0.1M NaOH in methanol. The solution was heated to 50 ˚C and stirred vigorously for 30 min then concentrated *via* rotory evaporation to yield a brown-purple solid. The solid residue was re-dissolved in 70 mL of D.I. H_2O and the resulting cloudy solution was passed through a 0.45 µM PTFE syringe filter. To the resulting clear, brown solution was added 1.064 mL of 2.0 M $Ba(OAc)_2$. After two minutes, an additional ppt formed which was removed *via* centrifugation (3,214 rcf, 5 min) and discarded. The supernatant was diluted 2x with Absolute EtOH and a thick white-brown ppt formed. This ppt was collected *via* centrifugation (3,214 rcf, 5 min) and washed with 1:1 H_2O:EtOH by 3 cycles of re-suspension and centrifugation (3,214 rcf, 5 min), discarding the supernatant after each cycle. This barium salt was re-suspended in D.I. H_2O with the assistance of sonication. After all large chunks were broken apart to yield a homogeneous mixture, the suspension was applied to a column of Dowex 50 Wx2 100-200 mesh cation exchange resin (0.6 mmol/mL wet bed volume) in H^+ form and eluted with 400 mL of D.I. H_2O. The eluent was collected in a receiving flask containing 0.50 mL (4.9 mmol) of TEA in 80 mL of EtOH. The

resulting yellow-brown solution was concentrated to a small volume (~20 mL) *via* rotory evaporation, then transferred to a tared glass vial, frozen in liquid N_2, and lyophilized. The title compound was obtained as 0.6532 g (1.231 mmol, 62.5%) of a glassy, yellow solid. ^{1}H NMR (300 MHz, D_2O, pD = 7.66) δ 8.26 (d, *J* = 1.51 Hz, 1H), 7.97 (dd, *J*1 = 1.53 Hz, *J*2 = 8.28 Hz, 1H), 7.87 (d, *J* = 8.29 Hz, 1H), 5.75 (dq, *J*1 = 6.37 Hz, *J*2 = 8.10 Hz, 1H), 4.05 (d, *J* = 2.42 Hz, 2H), 3.07 (q, *J* = 7.34 Hz, 12H), 2.54 (t, *J* = 2.44 Hz, 1H), 1.48 (d, *J* = 6.36 Hz, 3H), 1.15 (t, *J* = 7.33 Hz, 18H); ^{13}C NMR (75 MHz, D_2O, pD = 7.66) δ 168.1, 146.1, 145.2 (d, *J* = 3.09 Hz), 132.6, 132.1, 128.9, 123.5, 76.1, 76.0, 68.0 (d, *J* = 3.94 Hz), 46.4, 29.2, 23.9 (d, *J* = 5.21 Hz), 8.05; ^{31}P NMR (122 MHz, D_2O, pD = 7.66) δ 2.61 (d, *J* = 8.58 Hz). HRMS (*m/z*): $[M-H]^-$ calcd for $C_{12}H_{12}N_2O_7P^-$ 327.03876; found, 327.0391. $[2M-H]^-$ calcd for $C_{24}H_{25}N_4O_{14}P_2^-$ 655.08480; found, 655.0854.



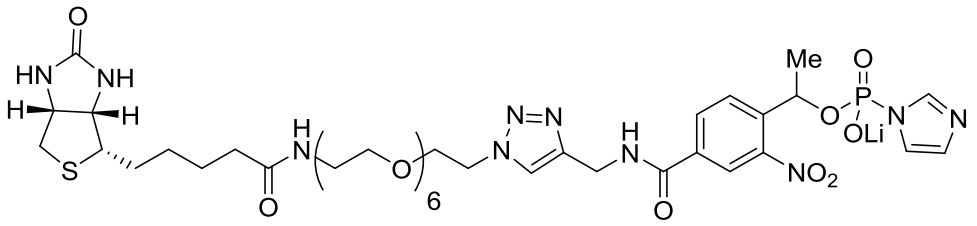
Biotin-PEG$_6$-Et-N$_3$ (12)

175.0 mg (0.716 mmol) of solid biotin and 228.2 mg (0.716 mmol) of solid HATU (CAS# 148893-10-1) were suspended in 1.43 mL of DMF with 237 µL (1.43 mmol) of neat DiPEA. The resulting white-yellow suspension gradually clarified with the assistance of vortexing over ~5 min. After standing for 5 min, the clear, yellow, activated biotin solution was added to a 228 mg (0.651 mmol) sample of neat *O*-(2-Aminoethyl)-*O′*-(2-azidoethyl)pentaethylene glycol (**20**) (CAS# 957486-82-7). The resulting solution was allowed to stir at r.t. for 1.5 h. 200 µL aliquots of the reaction solution were diluted into 3 mL aliquots of 1:1 ACN:H_2O, passed through a 0.45 µM PTFE syringe filter, and subject to preparatory HPLC purification using **Method 1** Since the product absorbs UV light poorly, fractions were assayed for the desired product by LCMS in order to determine the elution time. The title compound was obtained as 292.6 mg (0.507 mmol, 78 %) of a white, waxy solid. ^{1}H NMR (300 MHz, D_2O) δ 4.59 (dd, *J*1 = 4.83 Hz, *J*2 = 7.92 Hz, 1H), 4.31 (dd, *J*1 = 4.46 Hz, *J*2 = 7.92 Hz, 1H), 3.62 – 3.58 (m, 22H), 3.51 (t, *J* = 5.27 Hz, 2H), 3.39 (t, *J* = 5.07 Hz, 2H), 3.28 (t, *J* = 5.27 Hz, 2H), 3.24 – 3.19 (m, 1H), 2.88 (dd, *J*1 = 4.93 Hz, 13.06 Hz, 1H), 2.67 (d, *J* = 13.04, 1H), 2.16 (t, *J* = 7.22 Hz, 2H), 1.68 – 1.41 (m, 4H), 1.37 – 1.25 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 176.7, 165.2, 69.5 – 69.3 (estimated to account for 10 carbons in the PEG chain), 69.1, 68.7, 61.9, 60.1, 55.2, 50.0, 39.6, 38.8, 35.3, 27.8, 27.6, 25.0. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{24}H_{45}N_6O_8S^+$ 577.30141, found 577.2995; $[M+Na]^+$ calcd for $C_{24}H_{44}N_6NaO_8S^+$ 599.28335, found 599.2812. IR (dry film) $\overline{\nu}_{max}$: 2103 (N_3) cm^{-1}.



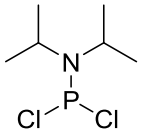
1-(2-nitro-4-(((1-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)carbamoyl)phenyl)ethyl dihydrogen phosphate (13)

The copper-mediated azide-alkyne cycloaddition reaction was performed following guidelines provided by M.G. Finn and coworkers[8]. To a round bottom-flask equipped with stir-bar was added 0.3679 g (0.6936 mmol) of solid alkyne **11.** 0.4000 g (0.6936 mmol) of solid Biotin-PEG_6-azide **12** was dissolved in 11.88 mL of D.I. H_2O and added to the flask. In a separate vial, 138.8 µL of aqueous 0.50 M $CuSO_4$ (69.4 µmol) was mixed with 694 µL of aqueous 100 mM THPTA (69.4 µmol) and the solution turned dark blue. The THPTA-Cu^{2+} complex solution was added to the solution of **11** and **12**. 1.156 mL of freshly prepared aqueous 300 mM sodium ascorbate (347 µmol) was added and the solution turned from blue to light brown. The flask was capped, purged with N_2 and allowed to stir under N_2 at r.t. overnight. The reaction was quenched with 1.00 mL of aqueous 0.1 M EDTA (pH 8.5), and passed through a 0.45 µM nylon syringe filter, rinsing forward with D.I. H_2O. The solution slowly turned green. The rxn mixture was purified *via* preparatory HPLC using **Method 2**. 0.628 g (0.694 mmol, 100%) of the title compound was obtained as a yellow liquid. ^{1}H NMR (300 MHz, D_2O, pD = 2.10) δ 8.34 (d, *J* = 1.17 Hz, 1H), 8.07 (dd, *J*1 = 0.92 Hz, *J2* = 8.39 Hz, 1H), 8.03 (s, 1H), 7.89 (d, *J* = 8.27 Hz, 1H), 5.86 (p, *J* = 6.38 Hz, 1H), 4.62 (s, 2H), 4.55 (t, *J* = 4.83 Hz, 2H), 4.48 (dd, *J*1 = 4.76 Hz, *J*2 = 7.89 Hz, 1H), 4.28 (dd, *J*1 = 4.42 Hz, *J*2 = 7.89 Hz, 1H), 3.87 (t, *J* = 4.79 Hz, 2H), 3.56 – 3.44 (m, 24H), 3.27 (t, *J* = 5.17 Hz, 2H), 3.16 (p, *J* = 4.41 Hz, 1H), 2.85 (dd, *J*1 = 4.91 Hz, *J*2 = 13.04 Hz, 1H), 2.64 (d, *J* = 12.95 Hz, 1H), 2.14 (t, *J* = 7.28 Hz, 2H), 1.63 – 1.37 (m, 4H estimated: integration occluded by doublet), 1.56 (d, *J* = 6.34 Hz, 3H estimated: integration occluded by multuplet), 1.32 – 1.22 (m, 2H); ^{13}C NMR (75 MHz, D_2O, pD = 2.10) δ 176.4, 166.9, 164.9, 146.4, 141.7 (d, *J* = 4.09 Hz), 133.6, 132.4, 128.6, 125.0, 123.5, 118.1, 70.6 (d, *J* = 4.47 Hz), 69.6 – 69.4 (estimated to account for 9 signals from PEG chain), 69.3, 68.8, 68.5, 61.94, 60.11, 55.3, 50.4, 39.6, 38.8, 35.3, 34.6, 27.9, 27.6, 25.1, 23.3 (d, *J* = 5.61 Hz). ^{31}P NMR (122 MHz, D_2O, pD = 2.10) δ -1.29 (d, *J* = 7.96 Hz). HRMS (*m/z*) $[M-H]^-$ calcd for $C_{36}H_{56}N_8O_{15}PS^-$ 903.33289; found 903.3330 $[M+H]^+$ calcd for $C_{36}H_{58}N_8O_{15}PS^+$ 905.34745; found 905.3508. IR (dry film) shows disappearance of azide absorption peak noted for compound **12**.



Lithium 1-(2-nitro-4-(((1-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)carbamoyl)phenyl)ethyl (1H-imidazol-1-yl)phosphonate (3)

To an r.b. flask equipped with stir-bar was added 0.251 g (0.277 mmol) of phosphate monoester **13** followed by 0.218 g (0.831 mmol) of solid PPh_3, 0.183 g (0.831 mmol) of solid 2,2'-DTDP, and 0.189 g (2.77 mmol) of solid imidazole. All solids were dissolved in 1.338 mL of DMF with 116 µL (0.831 mmol) of added TEA, and the resulting clear, yellow solution was allowed to stir at r.t. for 6 h. The reaction solution was then distributed equally into 20 tared glass vials. In a separate flask, 0.118 g (1.11 mmol) of $LiClO_4$ was dissolved in 6.7 mL of ACN and this solution was distributed equally into the 20 vials containing the reaction mixture. Additional ACN was added to each vial until a ppt formed (total of ~3mL per vial). The ppt was adhered to the bottom of each tube *via* centrifugation (3214 rcf, 10 min) and became a white-yellow gel in each case. The supernatants were discarded. The yellow gels were washed with ACN, then each was re-dissolved in the minimum amount (~100 µL) of MeOH and re-precipitated by diluting each aliquot to ~3 mL with ACN. The precipitates were again collected *via* centrifugation. The supernatants were discarded and the precipitates were dried *in vacuo* to remove residual ACN. 0.1418 g (0.1476 mmol, 53%) of the title compound was obtained as aliquots of a white-brown solid. The vials were sealed from moisture and stored at -20 ˚C until just prior to use. ^{1}H NMR (300 MHz, D_2O, pD = 11.5) δ 8.17 (d, *J* = 1.47 Hz, 1H), 7.92 (s, 1H), 7.88 (dd, *J*1 = 1.49 Hz, *J*2 = 8.26 Hz, 1H), 7.67 (d, *J* = 8.61 Hz, 1H), 7.48 (s, 1H), 6.84 (s, 1H), 6.63 (s, 1H), 5.68 (qd, *J*1 = 6.39 Hz, *J*2 = 8.78 Hz, 1H), 4.56 (s, 2H), 4.50 (t, *J* = 4.85 Hz, 2H), 4.45 (dd, *J*1 = 4.82 Hz, *J*2 = 7.95 Hz, 1H) 4.26 (dd, *J*1 = 4.45 Hz, *J*2 = 7.93 Hz, 1H), 3.84 (t, *J* = 4.96 Hz, 2H), 3.53 – 3.91 (m, 22H), 3.24 (t, *J* = 3.39 Hz, 2H), 3.14 (p, *J* = 4.47 Hz, 1H), 2.82 (dd, *J*1 = 4.94 Hz, *J*2 = 13.06 Hz, 1H), 2.61 (d, *J* = 13.03 Hz, 1H), 2.11 (t, *J* = 7.24 Hz, 2H), 1.62 – 1.36 (m, 4H estimated: integration occluded by doublet), 1.47 (d, *J* = 6.37 Hz, 3H estimated: integration occluded by multuplet), 1.28 – 1.18 (m, 2H); ^{13}C NMR (151 MHz, D_2O, pD = 11.5) δ 176.8, 167.4, 165.3, 146.3, 144.5, 140.9 (d, *J* = 2.37 Hz), 139.4 (d, *J* = 5.89 Hz), 133.8, 132.3, 128.9 (d, *J* = 10.80 Hz), 128.7, 124.5, 123.3, 119.9 (d, *J* = 6.13 Hz), 70.7 (d, *J* = 5.31 Hz), 69.71, 69.7 – 69.4 (m, estimated to account for 10 carbons in PEG chain), 68.9, 68.8, 62.1, 60.2, 55.3, 50.1, 39.7, 38.9, 35.5, 35.1, 27.9, 27.1, 25.1, 23.4 (d, *J* = 6.85 Hz); ^{31}P NMR (122 MHz, D_2O, pD = 11.5) δ -9.90 (d, *J* = 8.72 Hz). HRMS (*m/z*): $[M-Li+3H]^{2+}$ calcd for $C_{39}H_{61}N_{10}O_{14}PS^{2+}$ 478.19080; found, 478.1909; $[M-Li+H+Na]^+$ calcd for $C_{39}H_{59}N_{10}NaO_{14}PS^+$ 977.35627; found, 977.3541; $[M-Li+2Na]^+$ calcd for $C_{39}H_{58}N_{10}Na_2O_{14}PS^+$ 999.33822; found, 999.3365.



1,1-dichloro-N,N-diisopropylphosphanamine (19)

The following protocol is based on a procedure from Drent *et al*[9]. To a solution of Phosphorus (III) chloride (10.0 g, 72.8 mmol) in dry hexanes cooled to 0 ˚C in a dry flask equipped with a large stir-bar under N_2 was added neat diisopropylamine (14.7 g, 146 mmol) dropwise over 30 min. Large amounts of salt precipitated and the solution became a white, chunky suspension; the flask was swirled occasionally to aid mixing. After the diisopropylamine addition, the suspension was allowed to warm to rt and stir for a further 2.5 h. The suspension was filtered and the filter cake was rinsed forward extensively with hexanes (800 mL) while stirring the filter cake to re-suspend. The solution was partially concentrated *via* rotory evaporation to remove hexanes, and the remaining hazy green residue was distilled (5 torr, 100 – 106 ˚C) using a Kugelrohr distillation apparatus to yield 12.29 g of a clear, colorless liquid which solidified when cooled on ice. This material was used in the next reaction (see protocol for **4**) without further purification or characterization.

V. Peptide Synthesis and Characterization

General Information:

All peptides were synthesized on the solid phase using Rink Amide MBHA resin (Anaspec). Reactions were carried out at room temperature unless otherwise noted, in fritted polypropylene columns from Bio-Rad, fitted into an IDEX 4-way switching valve, using nitrogen for agitation and vacuum for draining solvent. Relative to resin loading, 5.0 molar equivalents of each amino acid, 4.9 molar equivalents of 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, CAS# 148893-10-1), 10.0 molar equivalents of *N*,*N*-diisopropylethylamine (DiPEA), and a volume of DMF required to attain a 0.5 M solution of HATU were used for each coupling cocktail. A commercially available ninhydrin test from Sigma-Aldrich was used. The following Fmoc-protected L-amino acids (Anaspec and Iris) were used:
Fmoc-Ala-OH
Fmoc-Asn(trt)-OH
Fmoc-Pro-OH
Fmoc-Ser[PO(OBzl)-OH]-OH

Fmoc-Trp(BOC)-OH
Fmoc-Cys(trt)-OH

See general information of chemical synthesis section for details of characterization techniques.

Standard Protocol A:
The pre-loaded resin was washed with DMF and DCM then swelled in DMF for 30 minutes. The following steps were performed for every cycle of amino acid coupling with one batch wash and two flow-through washes with DMF after each step. During each incubation, the resin was agitated with N_2.

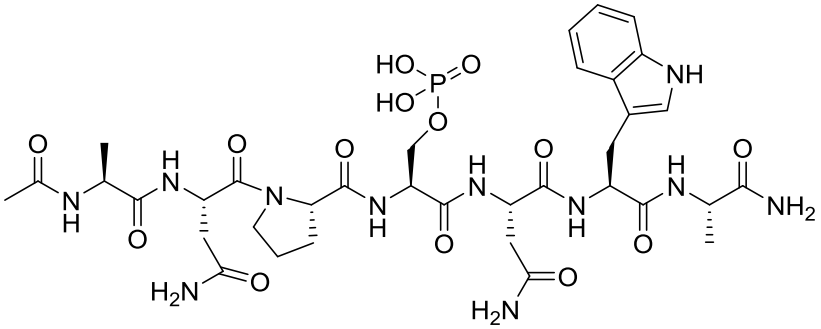
1. Fmoc deprotection: The resin was pre-incubated with 20% piperidine in DMF for 1 minute.
2. Fmoc deprotection: The resin was incubated with 20% piperidine in DMF for 20 minutes.
3. Amino Acid Coupling: The resin was incubated with a pre-mixed coupling cocktail containing the appropriate amino acid for 30 minutes or longer (up to 1 hour). Couplings known to fail repeatedly were heated to 55 ˚C in a water bath during coupling resin was agitated with a stir bar.
4. Ninhydrin Test: test was performed after each coupling to check that the reaction had gone to at least 98% conversion. If conversion was complete, the protocol was repeated starting from step 1; if incomplete, the protocol was repeated from step 3. If three couplings of the same amino acid failed to go to completion, the resin was acetylated and the synthesis continued from the next amino acid.
5. After coupling the final amino acid, the Fmoc group was removed by following steps 1 and 2.
6. Acetylation: The N-terminus was acetylated by performing 5 x 10 minute incubations with 20 molar equivalents of acetic anhydride and 40 molar equivalents of DiPEA. Additional rounds of acetylation were performed if the ninhydrin test indicated incomplete capping of the N-terminus.
7. The resin was washed with DCM and dried under vacuum aspiration.
8. The peptides were cleaved from the resin and deprotected using a standard TFA cleavage cocktail consisting of 95% TFA, 2.5% Milli-Q water, and 2.5% Triisopropylsilane. The cysteine-containing peptide was cleaved using a cocktail consisting of 95% TFA, 2 % Triisopropylsilane, 2% dithiothreitol, and 1% thioanisole. 10 μL of the cocktail was added for every milligram of peptide-loaded resin. The resulting orange-purple suspension was placed on a shaker at room temperature for 2.5 to 4 hours. *Initial test cleavages on small amounts of resin are recommended.
9. The cleavage cocktail was drained into a tared falcon tube, evaporated to small volume (approximately 10% of initial volume) under a stream of N_2, then precipitated using 30 mL of ice-cold diethyl ether. The peptide was collected by centrifugation (3x 4000 rpm, at 4 ˚C for 15 minutes, re-suspending in 30 mL of diethyl ether between spins) and the supernatant was discarded.

10. The pellet was dissolved in a minimum amount of Milli-Q water, frozen in liquid N_2, and lyophilized.
11. The crude, dry peptide pellet was weighed then submitted to preparatory HPLC for purification.

Standard Protocol B for Phosphopeptides:

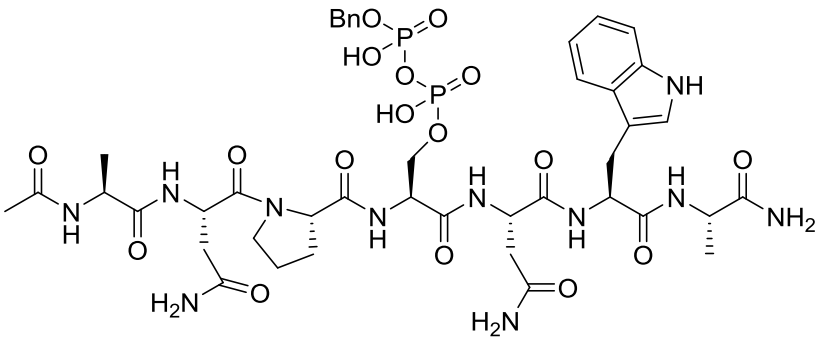
After incorporation of a phospho-amino acid into a growing peptide chain, steps 1 – 2 "Standard Protocol A" was modified to ensure complete removal of residual piperidine after each Fmoc deprotection step and to minimize the occurrence of β-elimination of the phosphate ester.

1. The resin was pre-incubated with 20% piperidine in DMF for 1 minute.
2. The resin was incubated with 20% piperidine in DMF for only 10 minutes to reduce base-mediated β-elimination.
3. The resin was washed with a solution of 5% DiPEA, 3x 1 minute to exchange piperidinium ions paired with the phosphate residue, followed by the standard wash protocol.
4. Starting "Standard Protocol A" was followed as above from Step 3 ("Amino Acid Coupling").

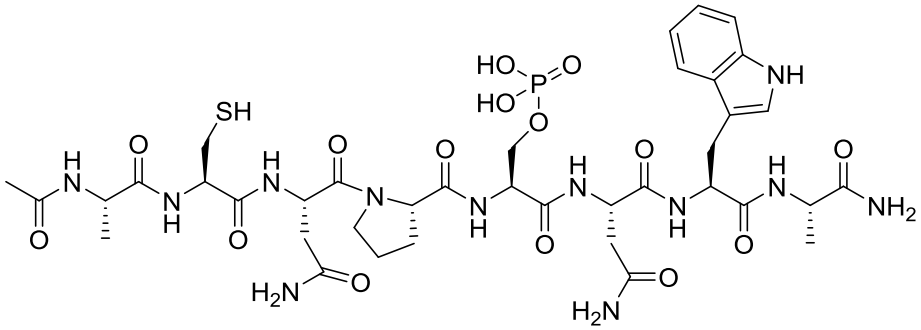


Ac-Ala-Asn-Pro-pSer-Asn-Trp-Ala-CO_2NH_2 phosphorylated model peptide (14)

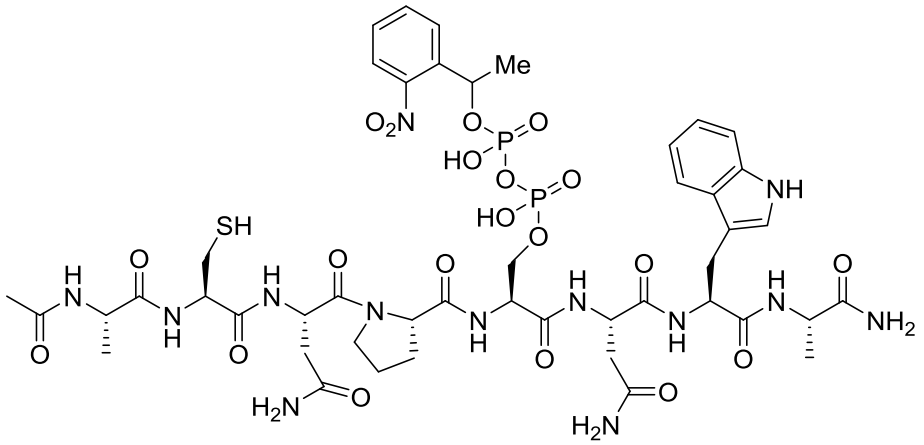
Model phosphopeptide **14** was synthesized on 802 mg of 0.71 mmol/g Fmoc-Rink Amide MBHA resin (570 µmol scale). A large fritted glass tube equipped with a three-way switching valve was used to accommodate the large amount of resin used for this synthesis. Standard Protocol A was used for the first four residues. The phosphoserine amino acid coupling was performed with half of the normal equivalents of each reagent and the coupling time was extended to 75 minutes. Standard Protocol B for phosphopeptide synthesis was used after the incorporation of phosphoserine. 324.5 mg of purified peptide was obtained (369 µmol, 65% yield) after preparatory HPLC purification using **Method 5**. LRMS (*m/z*): $[M+H]^+$ calcd for $C_{35}H_{51}N_{11}O_{14}P^+$ 880.33; found, 880.3. $[M+Na]^+$ calcd for $C_{35}H_{50}N_{11}NaO_{14}P^+$ 902.32; found 902.3. $[M-H+2Na]^+$ calcd for $C_{35}H_{49}N_{11}Na_2O_{14}P^+$ 924.30; found 924.3. Analytical HPLC **Method B** gives T_R = 8.531 min; **Method A** gives T_R = 9.031 min.



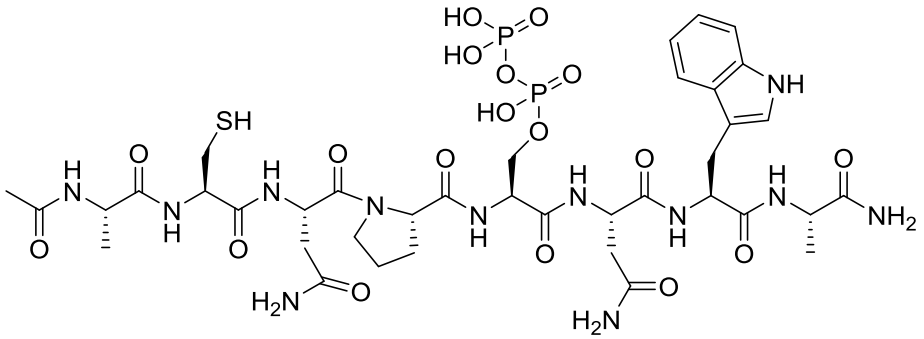
Ac-Ala-Asn-Pro-BnppSer-Asn-Trp-Ala-CONH$_2$ pyrophosphorylated model peptide (15) Pyrophosphorylated model peptide **15** was produced during the course of the standard kinetics experiments. ^{31}P NMR (203 MHz, DMA with 76 mM $ZnCl_2$ with internal $P(CH_3)_4Br$ in D_2O) δ -10.34 (b, 1P), -10.75 (b, 1P). ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{42}H_{59}N_{11}O_{17}P_2^+$ 1050.35; found 1050.3. $[M+2H]^{2+}$ calcd for $C_{42}H_{59}N_{11}O_{17}P_2^{2+}$ 525.68; found 525.7. Analytical HPLC **Method A** gives T_R = 9.850 min.



Ac-Ala-Cys-Asn-Pro-pSer-Asn-Trp-Ala-CONH$_2$ phosphorylated model peptide (21) Model cysteine-containing phosphopeptide **21** was synthesized on 378.5 mg of 0.71 mmol/g Fmoc-Rink Amide MBHA resin (269 µmol scale). Standard Protocol A was used for the first four residues. The phospho-serine amino acid coupling was performed with half of the normal equivalents of each reagent and the coupling time was extended to 140 min. Standard Protocol B for phosphopeptide synthesis was used after the incorporation of phospho-serine. The Asn residue coupled after proline was coupled 2x for 110 min. 56.1 mg of purified peptide was obtained (57.1 µmol, 21.2% yield) after preparatory HPLC purification using **Method 3**. LRMS (*m/z*): $[M+H]^+$ calcd for $C_{38}H_{56}N_{12}O_{15}PS^+$ 983.34; found, 983.3. Analytical HPLC **Method C** gives T_R = 9.194 min.



Ac-Ala-Cys-Asn-Pro-NPEppSer-Asn-Trp-Ala-CONH$_2$ NPE-protected pyrophosphorylated model peptide (16) A solid sample of reagent **2** (13.9 mg, 45.8 µmol) was suspended in 657 µL of 80 mM $ZnCl_2$ in DMA and the resulting suspension was sonicated to obtain a clear, light-yellow solution. A solid sample of cysteine model peptide **21** was dissolved in 605 µL of neat DMA to yield a clear, colorless solution. The P-imidazolide and peptide solutions were combined and the resulting clear, light-yellow solution was heated to 45 ˚C and allowed to stir for 90 min. The reaction solution was submitted directly to preparatory HPLC purification using **Method 4**. 15.6 mg (12.9 µmol, 84.3% yield) of the title compound was obtained as a yellow solid. ^{31}P NMR (H-decoupled, 203 MHz, D_2O, pD 8.78 with internal $P(CH_3)_4Br$ in D_2O) δ -11.67 (dd, *J*1 = 6.42 Hz, *J*2 = 20.43 Hz, 1P), - 12.23 (d, *J* = 20.42 Hz, 1P). LRMS (*m/z*): $[M+H]^+$ calcd for $C_{46}H_{64}N_{13}O_{20}P_2S^+$ 1212.36; found 1212.3; $[M+2H]^{+2}$ calcd for 606.68; found 606.8. Analytical HPLC **Method B** gives T_R = 10.819 min.



Ac-Ala-Cys-Asn-Pro-ppSer-Asn-Trp-Ala-CONH$_2$ pyrophosphorylated model peptide (17) The NPE-protected pyrophosphorylated model peptide **16** (15.6 mg, 12.9 µmol) was dissolved in 300 µL of Milli-Q H_2O and irradiated at 355 nm for 20 min in an Eppendorf tube. Progress of the photolysis reaction was monitored by analytical HPLC **Method B** (Product T_R = 9.151 min). The resulting solution was submitted directly to preparatory HPLC purification using **Method 4**. 5.5 mg (5.2 µmol, 40% yield) of a white solid were obtained. ^{31}P NMR (H-decoupled, 203 MHz, D_2O, pD 8.13 with internal $P(CH_3)_4Br$ in D_2O) δ -6.41 (d, *J* = 21.09 Hz, 1P), -10.83 (d, *J* = 22.14 Hz, 1P). LRMS (*m/z*): $[M+H]^+$ calcd for $C_{38}H_{57}N_{12}O_{18}P_2S^+$ 1063.31; found 1063.7.

VI. HPLC Methods

Analytical HPLC was performed using an Agilent 1260 Infinity Quaternary LC system with quaternary solvent delivery pump, an auto sampler, a diode-array detector, and a fraction collector. Solvents: **A** = 0.1% TFA in Milli-Q H_2O; **B** = 0.1% TFA in ACN.

- **Method A**: Equilibration with 10% solvent **B** in a solution of solvent **A** for 3 min followed by a linear gradient from 10% to 42% **B** over 8 min; 42-90% **B** over 1 min; 90% **B** isocratically for 1.5 min; 90% **B** to 10% **B** over 0.5 min with a flow rate of 1.00 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (100Å, 150 x 4.60 mm). Monitored 214 nm, 254nm, and 274 nm.
- **Method B:** Equilibration with 10% solvent **B** in a solution of solvent **A** for 3 min followed by a linear gradient of 10-90% solvent **B** over 20 min; 90% **B** isocratically for 1 min with a flow rate of 1 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (100Å, 150 x 4.60 mm). Monitored 214 nm, 254 nm, and 274 nm.
- **Method C:** Equilibration with 10% solvent **B** in a solution of solvent **A** for 3 min followed by a linear gradient of 10-50% solvent **B** over 10 min; 90% **B** isocratically for 1.5 min; 90% **B** to 10% **B** over 0.5 min with a flow rate of 1 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (100Å, 150 x 4.60 mm). Monitored 214 nm, 254 nm, and 274 nm.

Preparatory HPLC was performed on a Varian system with an SD-1 prep solvent delivery system, a ProStar 325 UV-Vis detector, and a 440-LC fraction collector. Solvents: **A** = 0.1% TFA in Milli-Q H_2O; **B** = 0.1% TFA in ACN; **C** = 0.1% FA in Milli-Q H_2O; **D** = 0.1% FA in ACN.

- **Method 1**: Equilibration with 10% solvent **D** in a solution of solvent **C** for 1.5 min followed by a linear gradient of 10-25% solvent **D** over 2.5 min; 25-35% solvent **D** over 5.5 min; 35-90% solvent **D** over 1 min; 90% solvent **D** isocratically for 3 min with a flow rate of 20 mL/min throughout. Used a Waters XBridge^{TM} 5µm C18 column (150 x 19mm). Monitored 254 nm.
- **Method 2**: Equilibration with 30% solvent **B** in a solution of solvent **A** for 2 min followed by a linear gradient of 30-50% solvent **B** over 8 min; 50-90% solvent **B** over 0.5 min; 90% solvent **B** isocratically for 0.5min; 90-30% solvent **B** over 1 min; 30% solvent **B** isocratically for 1 min with a flow rate of 21 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (21.2 x 250 mm). Monitored 254 nm.
- **Method 3**: Equilibration with 15% solvent **B** in a solution of solvent **A** for 1 min followed by a linear gradient of 15-42% solvent **B** over 12 min; 42-90% solvent **B** over 0.5 min; 90% solvent **B** isocratically for 1 min; 90-15% solvent **B** over 0.5 min with a flow rate of 21 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (21.2 x 250 mm). Monitored 254 nm.
- **Method 4**: Equilibration with 10% solvent **B** in a solution of solvent **A** for 2 min followed by a linear gradient of 10-90% solvent **B** over 20 min, then 90% solvent **B** isocratically for

2 min with a flow rate of 21 mL/min throughout. Used a Phenomenex Luna^{TM} 3µm C8(2) column (21.2 x 250 mm). Monitored 254 nm.

- **Method 5**: Equilibration with 10% solvent **B** in a solution of solvent **A** for 1 min followed by a linear gradient from 10% to 35% **B** over 4 in; 35% to 45% **B** over 5 min; 45% to 90% **B** over 2 min; 90% **B** isocratically for 1.5 min; 90% to 10% **B** over 0.5 min with a flow rate of 21 mL/min throughout. Used a Phenomenex LunaTM 3µm C8(2) column (21.2 x 250 mm). Monitored 254 nm.

VII. Kinetics Experimental Protocols and Data Analysis

General Information and Instrumentation

Samples of phosphorimidazolide reagent **1** and phosphopeptide **14** were weighed on a Mettler Toledo AT21 Comparator balance with a weighing capacity of 22 grams, a readability of 1 µg, and a linearity of 5 µg with a load of 5 grams. Liquid transfers were performed with Finnpipette F1 pipettes from Thermo Scientific with capacities of 2-20 µL, 10-100 µL, 20-200 µL, and 100-1000 µL. DMA solutions, and aqueous solutions were passed through 0.45 µm PTFE syringe filters before use.

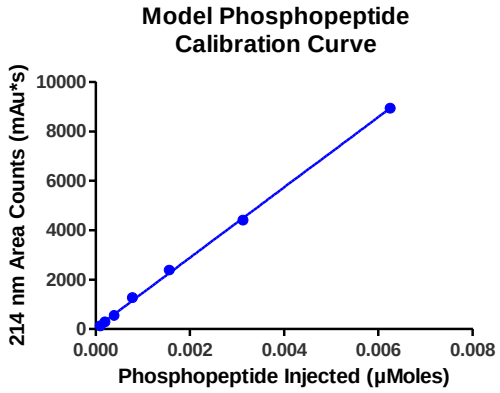
Kinetics, General Procedure:

Solid $ZnCl_2$ was dissolved in a sufficient volume of the necessary solvent to provide a solution of $ZnCl_2$ at the concentration desired for the reaction. A solid sample of Lithium monobenzylphosphorimidazolide (**1**) with a precisely known mass of between 10.0 mg and 15.0 mg was suspended in the volume of the necessary solvent with pre-dissolved $ZnCl_2$ to provide the desired final concentration of phosphorimidazolide reagent. Over the course of 3 – 5 minutes with the assistance of sonication, the phosphorimidazolide reagent dissolved to yield a clear, colorless solution. This solution was pre-warmed to the appropriate reaction temperature in a water bath for 5 minutes. To start the reaction, an aliquot of the phosphorimidazolide and $ZnCl_2$ stock solution was added to a solid lyophilized sample of phosphopeptide **14**, with a precisely known mass of at least 1.00 mg, in a vial equipped with stir bar, and the reaction timer was simultaneously started. The volume of the solution containing P-imidazolide and $ZnCl_2$ used was chosen to provide the desired initial concentration of phosphopeptide **14**. For all pseudo-first order kinetics experiments, the initial concentration of phosphorimidazolide reagent **1** was 100 mM, phosphopeptide **14** was 10 mM, and $ZnCl_2$ was 267 mM. For all reaction progress kinetics experiments, the initial concentration of reagent **1** was 30 mM, phosphopeptide **14** was 10 mM, and $ZnCl_2$ was 80 mM. The peptide dissolved immediately in all reaction solvents used. At various time-points, 10.0 µL samples of the clear, colorless reaction solution were quenched into 90.0 µL aliquots of aqueous

0.1 M EDTA (pH = 8.5) to provide 10x dilutions of the reaction mixture. Each time-point aliquot was submitted to HPLC analysis using **Method A**. The analytical HPLC auto-sampler injected 5.0 µL or 10.0 µL of each sample for each analysis, and the calculation for peptide concentration in the sample was adjusted accordingly.

Kinetics Data Analysis

The area under the peak from phosphopeptide **14** from each 214 nm HPLC chromatogram was converted to moles of phosphopeptide injected onto the HPLC column using the calibration curve below and then used to calculate the concentration in the reaction solution using HPLC injection volume and time-point dilution values.



Best-fit values	
Slope	1.4219e+006 ± 16472
Y-intercept when X=0.0	47.383 ± 44.930
X-intercept when Y=0.0	-3.3323e-005
1/slope	7.0328e-007
95% Confidence Intervals	
Slope	1.3796e+006 to 1.4643e+006
Y-intercept when X=0.0	-68.132 to 162.90
X-intercept when Y=0.0	-0.00011687 to 4.7013e-005
Goodness of Fit	
R square	0.99933
Sy.x	90.381

Calibration curve for model phosphopeptide (**14**) using analytical HPLC **Method A**.

Pseudo First-Order Kinetics Analysis

Phosphopeptide concentration vs. time data was converted to Ln(concentration (M)) vs. time (sec). A linear fit of the plot was found using Graph Pad Prism software version 5.04: the slope corresponds to the negative observed reaction rate ($-k_{obs}$). To find the second-order rate constant k of the reaction, this value was divided by the concentration of P-imidazolide **1** and entered into **Table 1**.

Reaction Progress Kinetics Analysis (RPKA)

Using Graph Pad Prism software version 5.04, one-phase decay curves were fitted to plots of phosphopeptide **14** concentration (M) vs. reaction time (sec). One-phase decay curves have the form given by **Equation 1** in which: $\boldsymbol{y_0}$ is the initial phosphopeptide concentration, $\boldsymbol{P}$ is the

calculated plateau value of peptide concentration at infinite time, and $\boldsymbol{k_{obs}}$ is the observed rate constant expressed in sec^{-1}.

$$\textbf{(1)} \quad \boldsymbol{y = (y_0 - P)e^{-k_{obs}t} - P}$$

The rate of the reaction at time $\boldsymbol{t}$ was calculated by taking the derivative of **Equation 1** to give **Equation 2**.

$$\textbf{(2)} \quad \boldsymbol{\frac{dy}{dt} = -k_{obs}t(y_0 - P)e^{-k_{obs}t}}$$

To calculate the phosphorimidazolide reagent concentration ($[Pimidazolide]_t$) at any given reaction time t, the assumption was made that the only significant mode of consumption of the reagent over the time-course of the reaction was *via* reaction with the model phosphopeptide. This is supported by the observation that the half-life of reagent **1** in 1:9 H_2O:DMA at 45 °C is 13 hours. Thus, **Equation 3** was used to calculate phosphorimidazolide reagent concentration at time t based on the initial concentration of the phosphorimidazolide reagent **1** ($[Pimidazolide]_i$), the initial concentration of the phosphopeptide **14** ($[Ppeptide]_i$), and on the phosphopeptide concentration measured by analytical HPLC at time t ($[Ppeptide]_t$).

$$\textbf{(3)} \quad [Pimidazolide]_t = [Pimidazolide]_i - ([Ppeptide]_i - [Ppeptide]_t)$$

To determine the reaction rate constant, the molar concentration of phosphopeptide at time t was plotted against the normalized reaction rate (the reaction rate at time t divided by the phosphorimidazolide reagent concentration at that time) (**Figure S8**). The slope of the resulting line is the second order rate constant $\boldsymbol{k}$ in units of $M^{-1}sec^{-1}$ entered into **Table S2**.

VIII. Protocols for DNA Manipulation, Cell Culture, Gel Electrophoresis, and Protein Expression

General Information and Instrumentation

Absorption spectra of DNA aliquots and $O.D._{600nm}$ measurements of bacterial cultures were taken with a NanoDrop 2000c spectrophotometer from Thermo Scientific (data analysis with NanoDrop 2000c software version 1.4.1). Plasmid DNA minipreps were performed with a QIAprep® Spin Miniprep Kit from Qiagen GmbH. Milli-Q water from a Sartorius stedum arum® 611UV dispenser was used to prepare media and buffer solutions. LB media, TB media, and agar were obtained as

dry powders from Carl Roth GmbH. DH5α electrocompetent cells for plasmid storage were purchased from New England Biolabs. BL21Δ*serB* cells were provided by the laboratory of Jason W. Chin[10]. Antibiotics were used at the following concentrations: Chloramphenicol: 25 µg/mL; Spectinomycin: 50 µg/mL

Lysis Buffer (pH 7.4):
154 mM NaCl, 10 mM Na_2HPO_4, 20 mM Imidzole, 0.20 mM PMSF, 0.5 mg/mL Lysozyme, 1 Unit/mL of DNAse I

Phosphate-Buffered Saline (PBS, pH 7.4):
154 mM NaCl, 10 mM Na_2HPO_4

SOC Outgrowth Medium:
2% Vegetable Peptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM MgSO4, 20 mM Glucose (Provided by New England Biolabs)

Gel Electrophoresis and Staining

Protein samples were mixed with 4x Laemmli sample buffer (Bio-Rad) containing 10% β-mercaptoethanol, heated to 95 °C for 8 min, then cooled and consolidated by centrifugation (5000 rpm, 1min) before gel loading. Bio-Rad Mini-Protean TGX Stain-Free precast 4-20% gradient gels with 10x 30 µL wells were used. Gels were run using a Bio-Rad PowerPac HC 300W power source set to 150 V for 60 minutes or until the loading dye ran off the gel. Gels were washed with D.I. H_2O 3x 5 min, then stained for 1 h with GelCode™ Blue colloidal coomassie stain G-250 from ThermoFisher Scientific. Gels were de-stained by multiple D.I. water washes.

Plasmids

1. pKW2 EF-Sep ($camp^R$) – encodes amber codon suppression system: modified aminoacyl tRNA synthetase SepRS(2), pSer incorporating tRNA(B4)$^{Sep}_{CUA}$, and the elongation factor engineered to accommodate pSer: EF-Sep[10].
2. pCDF Myo-127TAG ($spec^R$) – encodes Myo-D127pS (with amber stop codon) under control of the arabinose promoter.
3. pCDF Myo-wt ($spec^R$) – encodes Myo-wt under control of the arabinose promoter.

Mutagenesis Primers

Myo pS127 → D127 mutagenesis primer (TAG to GAC). Performed using Phusion HF DNA polymerase with 30 sec annealing at 68 ˚C.
Forward: 5'- cttcggtgctGACgctcagggtgctatgaacaaagc -3'
Reverse: 5'- caccctgagcGTCagcaccgaagtcacctggatgtctag-3'

Myo G154 → C154 mutagenesis primers (GGT to TGT). Performed using Phusion HF DNA polymerase with 3 min annealing at 72 ˚C.
Forward: 5'- caaagaactgggttaccagTGTggctcgggacatcatc -3'
Reverse: 5'- gatgatgtcccgagccACActggtaacccagttctttg -3'

Sequencing Primer

pCDF Myo-wt and pCDF Myo-127TAG:
Forward: 5'- cccgatcaaatacctggaattca-3'

Electrocompetent Cell Preparation

This protocol was adapted from "Introduction of Plasmid DNA into Cells" from Current Protocols in Molecular Biology, 1997[11]. A single colony of BL21 Δ*serB* was picked from an LB-Agar plate, inoculated into a 5 mL culture of LB media and incubated at 37 ˚C, and 180 rpm overnight. The 5 mL culture was inoculated into 300 mL of LB media with the appropriate antibiotic(s) and incubated at 180 rpm for 2 hours. The culture was allowed to reach an OD_{600} of 0.45 (target = 0.35 – 0.40 for early to middle log growth phase). The culture was cooled on ice for 15 min, and then split between six pre-chilled 50 mL centrifuge tubes. From this point on, the cells were maintained at a temperature of 4 ˚C. The cells were centrifuged 3 x (2400 rpm, 4 ˚C, 20 min) discarding supernatant after each spin and re-suspending each pellet in 40 mL 4 ˚C Milli-Q H_2O and 20 mL 4 ˚C Milli-Q H_2O after the first and second spins respectively. After the third spin, each pellet was re-suspended in 4 mL of 10% glycerol in Milli-Q H_2O at 4 ˚C and transferred to pre-chilled 15 mL centrifuge tubes. The cells were centrifuged as before and supernatant was removed. The two cell pellets were each re-suspended in 500 µL of pre-chilled 10% glycerol in Milli-Q H_2O. 100 µL aliquots of the cell suspensions were distributed into pre-cooled sterile 1.5 mL micro centrifuge tubes and stored at -80 ˚C until ready for use.

Plasmid Transformation by Electroporation

This protocol was adapted from "Introduction of Plasmid DNA into Cells" from Current Protocols in Molecular Biology, 1997[11] and Electroporation Tips from New England Biolabs Inc..[12] The following items were thawed or pre-chilled on ice:

- Electrocompetent cell aliquots
- Sterile 1.5 mL Eppendorf tubes
- Electroporation cuvettes with 1.0 mm gaps for *E. coli*. (Bio Rad Catalogue # 165-2089)
- DNA plasmids

The following items were pre-warmed to 37 ˚C:

- LB agar plates with the appropriate antibiotics
- Sterile 5 mL culture tubes
- SOC outgrowth medium

25 µL aliquots of thawed and re-suspended electrocompetent cells were transferred to electroporation cuvettes, taking care to evenly distribute the aliquots along the bottoms of the cuvettes. 1.0 µL aliquots of each DNA plasmid to be transformed were transferred to 25 µL aliquots of electrocompetent cells, taking care to avoid introducing air bubbles. Each cell and DNA aliquot was pulsed on a Gene Pulser X-cell system from BioRad using the default settings for *E. coli* (Voltage: 1800 V, Capacitance: 25 µF, Resistance: 200 Ω, Gap length: 1.0mm). (Actual pulse times were 4.6 – 5.1 ms; actual voltages were 1786 – 1788 V.) 975 µL of pre-warmed SOC outgrowth medium was transferred to each cuvette less than 30 seconds after pulsing. The resulting suspensions of electroporated cells were transferred to pre-warmed 5 mL culture tubes[‡] and incubated at 37 ˚C, 200 rpm for 1 hour. Typically 100 µL or less of the recovered cell suspensions were inoculated onto pre-warmed LB plates with the appropriate antibiotics.

Protein Expression

A single colony of BL21 Δ*serB* containing both the pKW2 EF-Sep plasmid ($camp^R$) and a plasmid encoding the protein to be expressed ($spec^R$) was inoculated into 5 mL of LB medium and incubated overnight at 37 ˚C, 200 rpm. 1.0 L of TB medium with chloramphenicol and spectinomycin was inoculated with this overnight culture and incubated at 37 ˚C, 220 rpm in a 4000 mL trident Erlenmeyer flask. Optical density was monitored at 600 nm. When the $O.D._{600\ nm}$ reached 0.6, the culture was induced by adding:

- 1.0 mL of 1.0 M IPTG (to a target concentration of 1.0 mM)
- 20 mL of 10% Arabinose (to a target concentration of 0.2% Arabinose)

[‡] Over-sized culture tubes were used to ensure vigorous agitation of the recovering bacteria.

- 16.3 mL of 125 mM (L)-*O*-phosphoserine (pH 7.0) (to a target concentration of 2.0 mM)

After a further 4 hours (37 ˚C, 200 rpm), the cultures were pelleted by centrifugation 1x (15 min, 3750 rcf, and 4 ˚C) and the supernatant was discarded. The pellets were re-suspended in sterile Milli-Q H_2O, chilled to 4 ˚C and distributed into 2x 50 mL centrifuge tubes. The cells were again pelleted by centrifugation as above. The supernatant was poured off, and the pellets[§] were stored at -80 ˚C.

IX. Purification and Characterization of Recombinantly Expressed Proteins

General Information

Cell lysis was performed with a Branson Digital Sonifier®, model 250. FPLC purification was performed on a Bio-Rad NGC™ Quest 10 Chromatography System equipped with a BioFrac fraction collector and controlled with ChromLab Standard Edition software, version 3.1.0.06. Dialysis was performed with a Spectra/Por® 7 dialysis membrane with 8 kDa MWCO or with Slide-A-Lyzer® Dialysis cassettes from Thermo Scientific with 2 kDa or 3.5 kDa MWCO and 0.5 – 3.0 mL capacity. Centrifugal spin concentrators (also referred to as spin desalting or spin filtration columns) were Merck-Millipore Amicon Ultra centrifuge filter units with volumes of 500 µL or 15 mL and MWCOs of 10 kDa. Gel images were taken with a Bio-Rad ChemiDoc™ CTD+ Molecular Imager® and processed with Image Lab software, version 5.2.1, build 11 from Bio-Rad. Protein concentrations were determined using a Pierce™ BCA Protein Assay Kit.

NiNTA Buffer A (loading buffer, pH 7.4):
154 mM NaCl, 10 mM Na_2HPO_4, 20 mM Imidazole

NiNTA Buffer B (elution buffer, pH 7.4):
154 mM NaCl, 10 mM Na_2HPO_4, 400 mM Imidazole

Cell Lysis and Lysate Processing

Pelleted cells[**] containing recombinantly expressed proteins were thawed in a water bath to rt, and each pellet was suspended in 25 mL of lysis buffer and lysed by sonication on ice (30% amplitude over 2 min with 2 sec bursts and 1 sec pauses) while maintaining the temperature of the suspension at or below 14 ˚C. The cell lysates were centrifuged (40,000 rcf, 30 min, 4 ˚C, with a JA-25.50

[§] For Myo expression, the cell pellets were red-brown.
[**] 1 pellet contains material from approximately 500 mL of induced culture

rotor) and the clarified supernatant[††] containing the recombinantly expressed protein was poured off. The supernatant was passed through a 0.45 µm nylon or PTFE whatman filter.

NiNTA FPLC Purification of Lysates

A 5 mL GE Healthcare HisTrap™ HP column with NiNTA resin was equilibrated with NiNTA Buffer A (PBS + 20 mM Imidazole). The clarified cell lysates containing the recombinantly expressed His_6-tagged protein of interest were loaded onto the column, and eluted with a gradient of increasing NiNTA Buffer B (PBS + 400 mM Imidazole) in Buffer A. The content of the protein fractions was visualized by SDS-PAGE. Product-containing fractions were combined, concentrated to a volume of ~5 mL using 10 kDa MWCO centrifugation filters (5000 rcf, 20 min, 4 ˚C, 15 mL capacity), and dialyzed against the appropriate starting buffer for ion exchange purification.

Myo-wt:

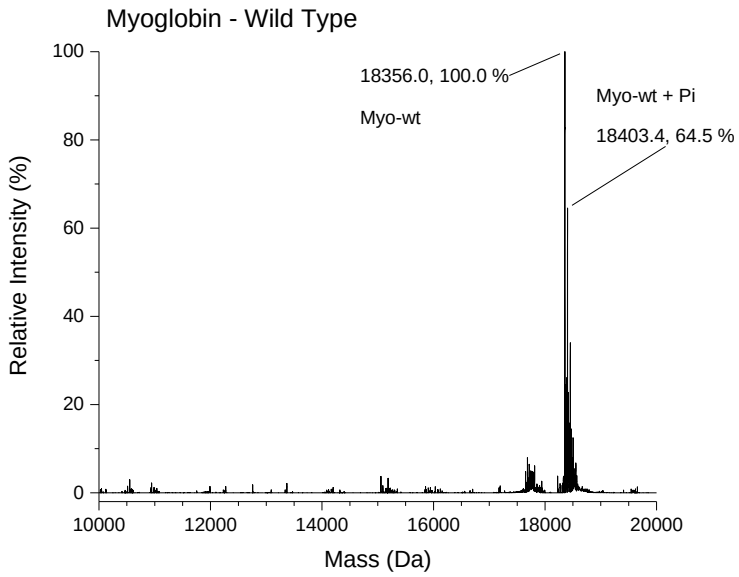
Strong cation exchange FPLC purification: A pre-packed 5 mL GE Healthcare Hi Trap™ SP HP cation exchange column was equilibrated with 25 mM MOPS and 15 mM NaCl in D.I. H_2O, pH 7.2. A gradient of 0-250 mM NaCl in 25 mM MOPS Buffer, pH 7.2 over 22 column volumes was applied. Monitored 280 nm. All red fractions were combined and concentrated to 1.65 mL in a 10 kDa MWCO centrifugation filter 2x (3214 rcf, 20 min, 15 ˚C, 15 mL capacity) without exchanging the buffer. BCA assay indicated a protein concentration of 2.56 mg/mL for a total yield of 4.59 mg of protein per liter of culture. The buffer was exchanged to 25 mM TRIS, 150 mM NaCl, pH 7.9 with 15% glycerol and aliquots were stored at -80 ˚C.

Sequence:

MVLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHG
VTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKA
LELFRKDIAAKYKELGYQGGSGHHHHHH

Calculated average molecular weight: 18355.07 Da

[††] For myoglobin expression, the supernatant was red-pink.



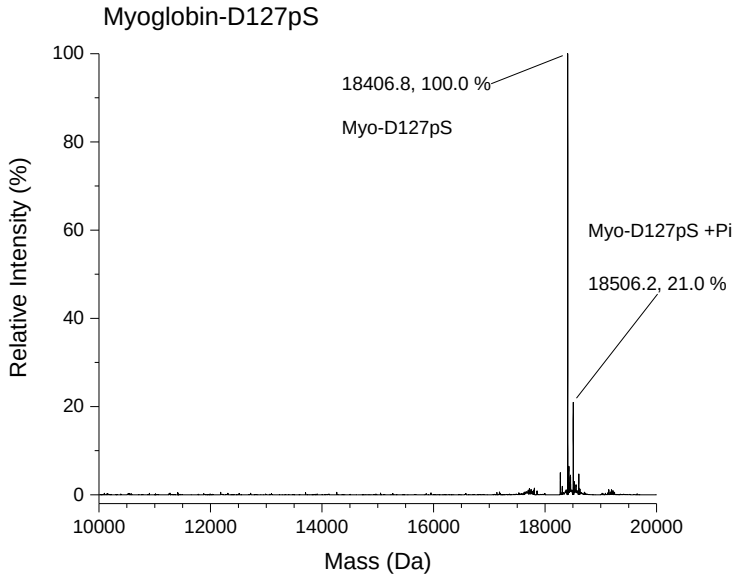
Myo-D127pS

Strong cation exchange FPLC purification: A pre-packed 5 mL GE Healthcare Hi Trap™ SP HP cation exchange column was equilibrated with 25 mM MOPS Buffer in D.I. H_2O, pH 7.2. A gradient of 0-250 mM NaCl in 25 mM MOPS Buffer, pH 7.2 over 11 column volumes was applied. Monitored 280 nm. Fractions corresponding to the two observed UV peaks were combined separately and concentrated using a 3 kDa MWCO centrifuge filter, 4x (5000 rcf, 20 min, 15 ˚C, 15 mL capacity), diluting to 15 mL with 25 mM TRIS, 150 mM NaCl (pH 7.9) between spins. Peak 1: 3.60 mL of 1.03 mg/mL protein for 3.71 mg of protein. Peak 2: 5.52 mL of 0.59 mg/mL protein for 3.25 mg of protein. Both proteins have MS spectra consistent with myoglobin but peak 1 is red, suggesting that peak 1 corresponds to holo-myoglobin while peak 2 corresponds to apo-myoglobin. Total yield was 6.96 mg protein per liter of culture. Proteins were stored at -80 °C.

Sequence:

MVLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHG
VTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGA(pS)AQGAMN
KALELFRKDIAAKYKELGYQGGSGHHHHHH

Calculated average molecular weight: 18407.04 Da.



Bottom-up proteomics confirmed high levels of phosphorylation at Ser127:

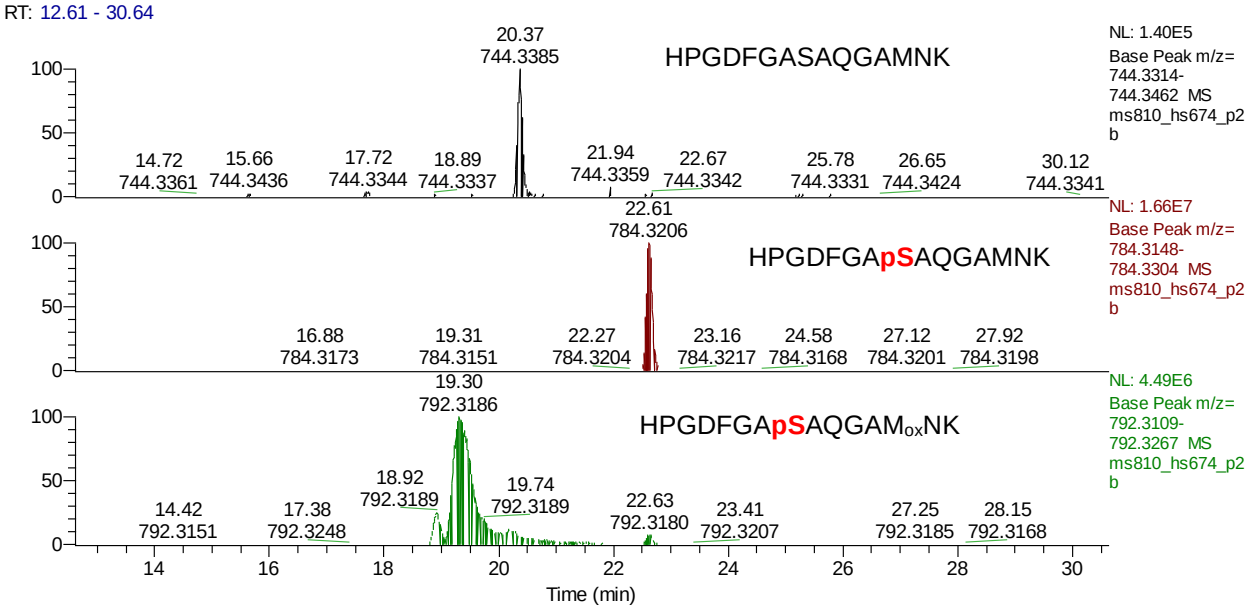


Figure S30. Single ion chromatograms with ion counts (NL:) of phosphorylated peptides from Myo-D127pS tryptic digest and MS/MS analysis. Ion abundances of 1.66 x10^7 to 4.49 x10^6 relative to the non-phosphorylated sequence (1.40 x10^5) indicate high levels of protein phosphorylation.

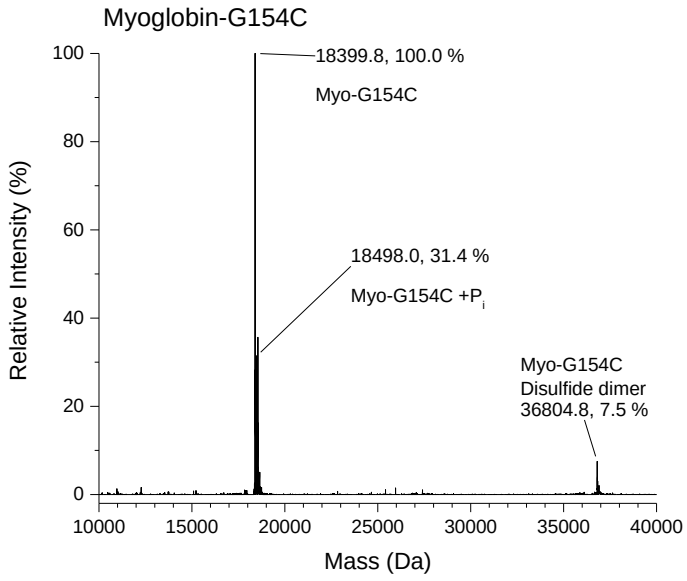
Myo-G154C

Strong cation exchange FPLC purification: A pre-packed 5 mL GE Healthcare Hi Trap™ SP HP cation exchange column was equilibrated with 25 mM MES and 2 mM DTT at pH 6.12. A gradient of 0 to 800 mM NaCl in 25 mM MES and 2 mM DTT Buffer, pH 6.12, over 22 column volumes was applied. Monitored 280 nm. SDS-PAGE of the resulting fractions indicated impure product. Product-containing fractions were combined and exchanged into a new cation exchange buffer. The pre-packed 5 mL GE Healthcare Hi Trap™ SP HP cation exchange column was equilibrated with 25 mM MOPS, 15 mM NaCl, 2.0 mM DTT at pH 7.2. A gradient of 15 to 300 mM NaCl with 25 mM MOPS, 2.0 mM DTT at pH 7.2 over 22 column volumes was applied. Monitored 280 nm. SDS-PAGE showed that red fractions contained pure product. Product fractions were combined and exchanged into 25 mM TRIS, 150 mM NaCl, 2.0 mM DTT with 15% glycerol at pH 7.9 and stored at -80 °C. 3.0 mL of 0.815 mg/mL protein were obtained for a yield of 2.4 mg of Myo-G154C per liter.

Sequence:

MVLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHG
VTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMN
KALELFRKDIAAKYKELGYQGCSGHHHHHH

Calculated average molecular weight: 18401.16 Da.



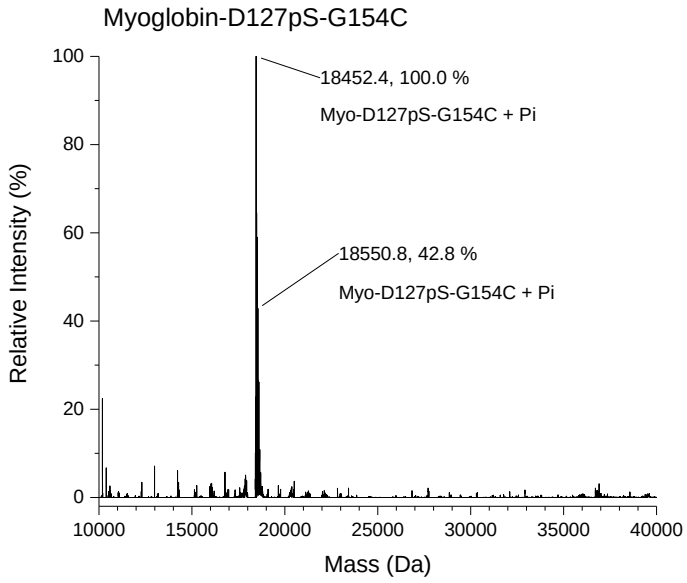
Myo-D127pS-G154C

Strong cation exchange FPLC purification: A pre-packed 5 mL GE Healthcare Hi Trap™ SP HP cation exchange column was equilibrated with 25 mM MES buffer at pH 6.12. A gradient of 0-800 mM NaCl in 25 mM MES Buffer, pH 6.12, over 12 column volumes was applied. Monitored 280 nm. UV-active fractions were visualized by SDS-PAGE and those with bands corresponding to pure product were combined, and exchanged into 25 mM TRIS, 150 mM NaCl, and 20 mM DTT (fresh), at pH 7.9 and incubated at 4 °C overnight to reduce disulfide dimers. The protein was then exchanged into storage buffer: 25 mM TRIS, 150 mM NaCl, 2.0 mM DTT, with 15% glycerol at pH 7.9 and stored at -80 °C. 4.97 mL of 0.108 mg/mL Myo-D127pS-G153C was obtained for a total of 0.537 mg per liter[‡‡].

Sequence:

MVLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHG
VTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGA(pS)AQGAMN
KALELFRKDIAAKYKELGYQGCSGHHHHHH

Calculated average molecular weight: 18453.13 Da.



Ub-wt

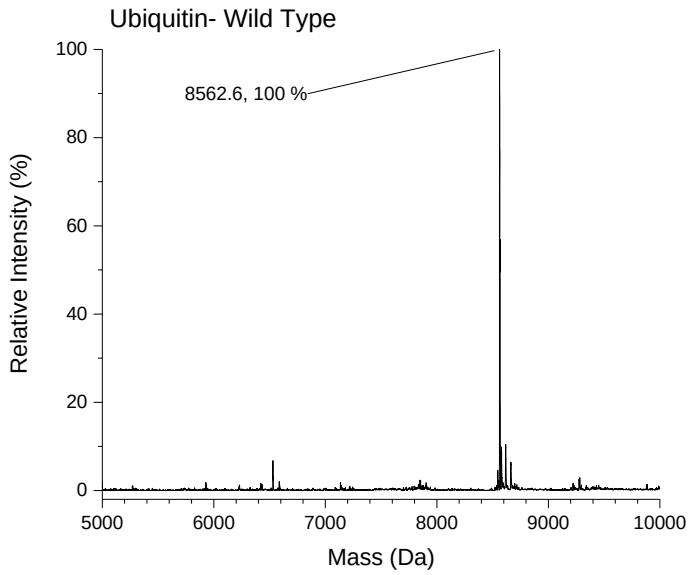
Recombinant human ubiquitin was purchased from BostonBiochem® (Cat # U-100H, Lot # 05011314-A) and used as provided.

[‡‡] Note that some protein was lost during an initial purification attempt.

Sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKEST
LHLVLRLRGG

Calculated average molecular weight: 8564.82 Da
ESI-MS of purified Ub-S65pS:



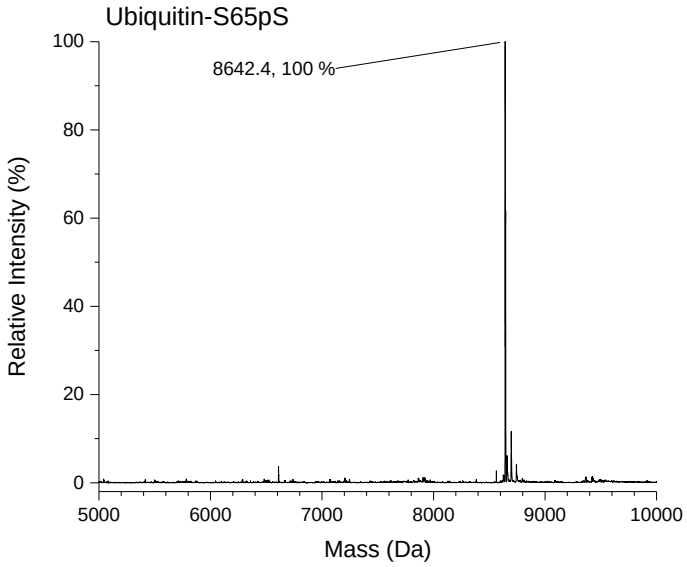
Ub-S65pS

Ub-S65pS was expressed and purified as described in D.T. Rogerson et al..[10]

Sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKE(pS)T
LHLVLRLRGG

Expected average molecular weight: 8644.08
ESI-MS of purified Ub-S65pS:



Bottom-up proteomics confirmed high levels of phosphorylation at Ser65:

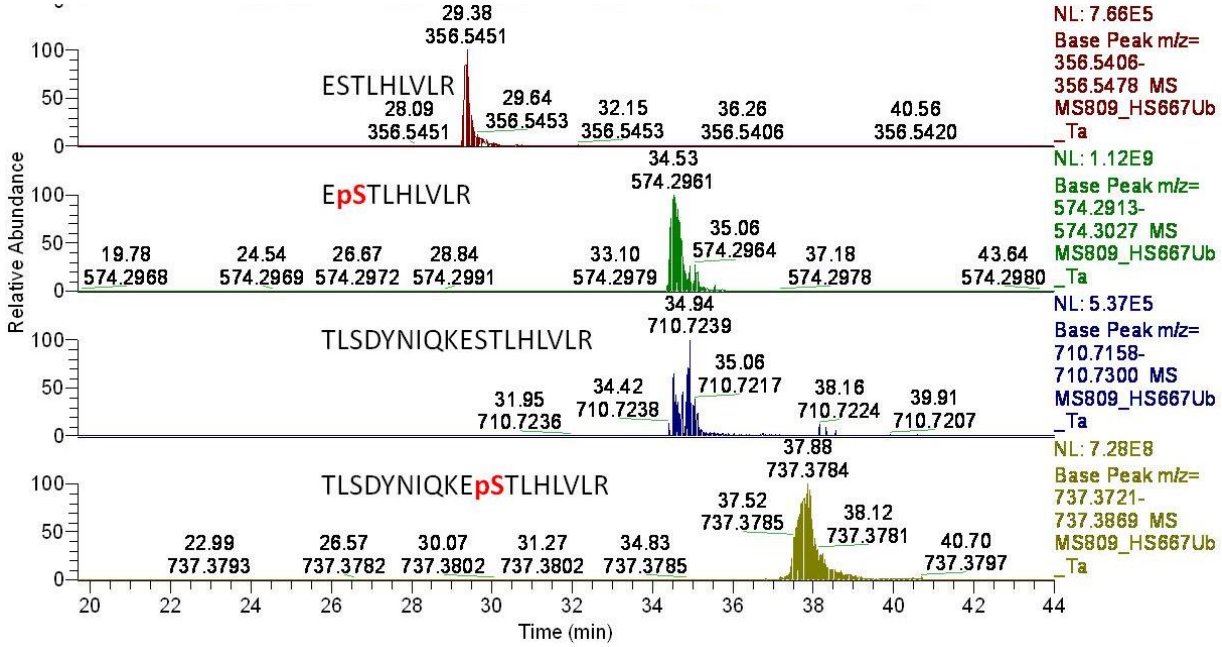


Figure S31. Single ion chromatograms with ion counts (NL:) of phosphorylated peptides from Ub-65pS tryptic digest and MS/MS analysis. Phosphorylated peptide ion abundances (7.28 x10^8 to 1.12 x10^9) relative to the non-phosphorylated sequences (5.37 x10^5 to 7.66 x10^5) indicate high levels of phosphorylation.

Protein Tryptic Digest

Protein aliquots were incubated at 37 ˚C for 7 hours in 50 mM NH_4HCO_3 (pH 7.8) with Sequencing Grade Modified Trypsin from Promega (part # qpIV511; reconstituted in 50 mM acetic acid) in accordance with the manufacturer's directions. Tryptic digests were stored at -20 ˚C until just prior to analysis.

Bottom-Up Proteomics Measurements

The digested myoglobin was dissolved in Milli-Q water and analyzed by a reversed-phase capillary liquid chromatography system (Dionex Ultimate 3000 NCS-3500RS Nano, Thermo Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Scientific). LC separations were performed on an in-house packed 75 µm inner diameter PicoTip™ column containing 25 cm of ReproSil-Pur® C18AQ resin (3 µm, 120 Å, Dr. Maisch GmbH Ammerbuch-Enttringen, Germany) at an eluent flow rate of 300 nL/min using a gradient of 2–50% B over 45 min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B 0.1% formic acid in acetonitrile. Fourier transform survey scans were acquired in a range from 350 to 1500 *m/z*, with a resolution of 120000 and an AGC target value of 2e5. Precursor ions with charge states of $2^+ – 4^+$ were isolated with a mass selecting quadrupole (isolation window *m/z* 1.6) and subjected to EThcD fragmentation. The dynamic exclusion time was 30 sec and the maximum injection time was set to 1sec to collect $1x10^5$ precursor ions. EThcD MS/MS spectra were acquired in the orbitrap with a resolution of 15000. EThcD fragmentation was performed using charge dependent ETD parameters and the supplemental activation was set to 50% for doubly charged precursor and 20% for higher charge states.

Bottom-Up Proteomics Analysis

The digested myoglobin was dissolved in Milli-Q water and analyzed by a reversed-phase capillary liquid chromatography system (Dionex Ultimate 3000 NCS-3500RS Nano, Thermo Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Scientific). LC separations were performed on an in-house packed 75 µm inner diameter PicoTip column containing 25 cm of ReproSil-Pur C18AQ resin (3 µm, 120 Å, Dr. Maisch GmbH Ammerbuch-Enttringen, Germany) at an eluent flow rate of 300 nL/min using a gradient of 2–40% B in 68 min. Mobile phase **A** contained 0.1% formic acid in water, and mobile phase **B** 0.1% formic acid in acetonitrile. FT survey scans were acquired in a range from 375 to 1500 m/z, with a resolution of 60000 and an AGC target value $4x10^5$. Precursor ions with charge states 2-4 were isolated with a mass selecting quadrupole (isolation window m/z 1.6) and subjected to EThcD fragmentation. The dynamic exclusion time was 30 sec and the maximum injection time was set to 1 sec to collect $5x10^4$

precursor ions. EThcD MS/MS spectra were acquired in the orbitrap with a resolution of 7500. EThcD fragmentation was performed using charge dependent ETD parameters and the supplemental activation was set to 30%.

Bottom-Up Proteomics Data Analysis

MS raw data were analyzed with Proteome Discoverer 2.1 software (Thermo Fisher Scientific, Bremen, Germany). The non-fragment filter was used with following parameters: Precursor ions and charged reduced precursors were removed within a 1 Da window and neutral losses within a 0.5 Da window. MS/MS spectra were searched against a database containing the myoglobin protein sequence and the yeast proteome using Sequest. Precursor mass tolerance and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively. Oxidation of methionine, phosphorylation (S, T, Y, C, K, H & R) and pyrophosphorylation (S & T) were searched as variable modifications. Percolator was used to filter peptide spectrum matches (PSMs) with a false-discovery rate (FDR) <0.05. PTM localization was performed by ptmRS (Version 3.0) using diagnostic ions and without PhosphoRS mode. Identified modified peptides were manually verified.

Intact Protein ESI-MS Measurements and Data Analysis

Intact proteins were analyzed using a reversed phase UPLC System (Acquity H class UPLC, Waters, Milford, USA) coupled to a Xevo G2-XS-Q-TOF instrument (Waters, Milford USA). Chromatographic separation was performed with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Component A of the mobile phase was 0.01% formic acid in water and component B was Acetonitrile with 0.01% formic acid. Separation was performed within 6 min *via* a linear gradient starting with 5% and ending with 95% of component B. Proteins were ionized in positive ion mode applying a spray voltage of 4 kV, a desolvation gas flow of 400 $L*min^{-1}$ and a source temperature of 100 °C. The data were acquired in sensitivity mode with a resolution R=20,000 (fwhm). Leucine-enkephalin (m/z 556.2771) was used for internal calibration. The generated raw data were deconvoluted with the MaxEnt 1 algorithm utilizing a resolution of 0.20002 Da for myoglobin samples and 0.10001 Da for ubiquitin samples.

X. Protocols for Protein Pyrophosphorylation, Photo-Release, and Further Characterization

General Information and Instrumentation:

The storage buffer for all protein aliquots used in pyrophosphorylation reactions was removed and exchanged for Milli-Q H_2O by at least 5 cycles of spin desalting. Amicon® Ultra Centrifugal Filters from Millipore Sigma with capacities of 15 mL or 500 µL were used for concentration and desalting of protein solutions. Steps which call for heating and agitation of protein samples were performed in an Eppendorf™ ThermoMixer®, equipped with a heated lid. Solutions made with DMA were passed through a 0.45 µm PTFE syringe filter before use. For affinity capture experiments, Streptavidin Sepharose High Performance resin from G.E. Healthcare Life Sciences with a loading of 300 nmol/µL was used. Photochemical reactions were conducted using an Atlas Photonics Lumos 43 light source with an optical output of 200 mW/cm^2.

General Protocol for Myoglobin Pyrophosphorylation:

To a solid sample of P-imidazolide reagent **1**, **2**, or **3** was added enough DMA with 340 mM $ZnCl_2$ to provide a 68.0 mM solution of the P-imidazolide reagent. The reagent dissolved to yield a clear solution over the course of a few minutes with the assistance of sonication. To a PCR tube containing a 5.0 µL aliquot of 9.0 µg/µL myoglobin in Milli-Q H_2O was added 45.0 µL of the freshly prepared P-imidazolide solution. The resulting clear red-brown solution (with 61.2 mM P-imidazolide, 49 µM protein and 306 mM $ZnCl_2$, in 1:9 H_2O:DMA) was incubated at 45 ˚C with 1000 rpm shaking for the desired reaction time. To achieve complete consumption of starting material, pyrophosphorylation reactions were conducted with reagent **1** for 100 min, reagent **2** for 180 min, or reagent **3** for 150 min. The 50 µL aliquot was quenched by dilution into 450 µL of aqueous 6 M guanidine HCl and allowed to stand for between 5 and 15 min at rt. This quenched solution was then rapidly diluted into a 50 mL solution of myoglobin refolding buffer consisting of 50 mM TRIS, 35 mM KCl, and 20 mM DTT in Milli-Q H_2O adjusted to pH 7.8. (Note that the solid DTT was dissolved in the refolding buffer no more than 3 hours before use to minimize oxidation.) The refolding solution was then allowed to stand at 4 ˚C overnight (14 – 20 hours) before being concentrated by multiple rounds of centrifugation in an 10 kDa MWCO centrifuge filter unit with a 15 mL capacity (20 min, 3,214 rcf, 17 ˚C) and then a 10 kDa MWCO centrifuge filter unit with a 500 µL capacity (20 min, 14,200 rcf, 17 ˚C). (Note that 10 kDa MWCO filters must be used when removing reagent **3**, as this construct does not pass through smaller MWCO filters.) After complete concentration (to a volume of 22 – 40 µL), the sample was exchanged into either PBS buffer or Milli-Q H_2O by four cycles of dilution with 430 µL of the desired solution followed by centrifugation (20 min, 14,200 rcf, 17 ˚C) in the 500 µL 10 kDa MWCO filter unit.

After the last cycle of centrifugation, the clear, red-brown concentrate was transferred to a tared sample tube to estimate the final volume by weight (assuming a density of 1mg/µL).

Seven 5.0 µL aliquots of 9.0 µg/µL Myo-D127pS were pyrophosphorylated with reagent **1** and refolded in parallel using this protocol, and the recovery of the combined refolded solutions was determined to be 30% using a Pierce™ BCA Protein Assay Kit.

Myoglobin Affinity Capture Experiment:

Four 5.0 µL aliquots of 9.0 µg/µL myoglobin were subject to the general myoglobin pyrophosphorylation protocol in 1:9 H_2O:DMA with 306 mM $ZnCl_2$ at 45 ˚C with a 2.5 hour reaction time. Samples:
1) Myo-wt, No P-imidazolide reagent
2) Myo-D127pS, No P-imidazolide reagent
3) Myo-wt, + reagent **3**
4) Myo-D127p, + reagent **3**

Separately, five 22.0 µL aliquots of re-suspended Streptavidin Sepharose High Performance resin (300 nmol/µL) were buffer-exchanged *via* three cycles of centrifugation (4 min, 10,000 rcf), supernatant removal, and re-suspension in 350 µL of PBS with 25 mg/mL albumin. The beads were incubated at 1200 rpm, rt for 40 min. The beads were washed to remove excess albumin *via* three cycles of centrifugation (4 min, 10,000 rcf), supernatant removal, and re-suspension in 400 µL of PBS buffer. The beads were incubated again (1200, rt) for 15 min, and washed two more times as above, then re-suspended in 250 µL of PBS after the final wash cycle.

After quenching, refolding, buffer exchanging, and concentrating[§§] to volumes of between 27 and 33 µL in PBS buffer, each protein sample was diluted 2x with PBS buffer and half of the total volume was then removed and added to a separate aliquot of the freshly-prepared albumin-blocked streptavidin beads. The streptavidin bead and protein aliquots were incubated at 1200 rpm, and rt for 60 min. Afterwards, the supernatant and three 400 µL PBS washes of each bead aliquot were combined in 10 kDa MWCO spin desalting filters and concentrated to final volumes of between 25 and 31µL.

21 µL aliquots of samples 1 – 4 and the supernatants of their corresponding bead aliquots were supplemented with 7.0 µL of Laemmli buffer (4x with 10% βME), heated to 95 ˚C for 8 min, cooled, and consolidated *via* centrifugation. 10 µL of a 250 µL aliquot of albumin-blocked

[§§] Note that protein samples must be thoroughly washed during the buffer exchange, as excess of reagent **3** can overwhelm the streptavidin beads and prevent efficient affinity capture of pyrophosphorylated proteins. **3** will not pass through filters with a smaller MWCO than 10 kDa.

streptavidin beads (not exposed to myoglobin) was also prepared for SDS-PAGE. 21 µL of each sample were loaded into separate lanes of a 4 – 20% Bio-Rad Mini Protean Gel (10x 30 µL wells) and run at 150 V for 60 min. The gel was washed with D.I. H_2O (3x 5 min) then stained with GelCode^{TM} Blue colloidal coomassie for 1 hour, and de-stained by repeated rinsing with D.I. H_2O.

Myoglobin Photo-Release:

Each of the four samples of streptavidin resin which had been exposed to myoglobin in the affinity capture experiment was subject to three new wash cycles consisting of: re-suspension in 400 µL PBS, centrifugation (10,000 rcf, 5 min), and removal of supernatant. Each aliquot was then re-suspended in 250 µL of PBS and irradiated with 360 nm light for three hours while stirring in a round-bottom Eppendorf tube equipped with a magnetic stir bar to maintain the beads in suspension. After irradiation, the bead suspensions were transferred to conical Eppendorf tubes, rinsing forward with 150 µL PBS. The supernatant of each bead aliquot and two bead washes (400 µL PBS) were combined and concentrated in separate 10 kDa MWCO spin desalting filters. Each sample of irradiated and washed streptavidin beads was re-suspended in 100 µL of PBS. 21 µL of each concentrated supernatant and re-suspended bead aliquot were supplemented with 7.0 µL of Laemmli buffer (4x with 10% βME), heated to 95 ˚C for 8 min, cooled, and consolidated *via* centrifugation. 21 µL of each sample were loaded into separate lanes of a 4 – 20% Bio-Rad Mini Protean Gel (10x 30 µL wells) and run at 150 V for 60 min. The gel was washed with D.I. H_2O (3x 5 min), fixed for 30 min with a solution of 50% methanol, 40% H_2O and 10% glacial acetic acid, stained with colloidal coomassie for 1 hour, then de-stained by repeated rinsing with D.I. H_2O.

Ubiquitin Pyrophosphorylation:

To a solid sample of P-imidazolide reagent **1** was added enough DMA with 340 mM $ZnCl_2$ to provide a 68.0 mM solution of the P-imidazolide reagent. The reagent dissolved to yield a clear solution over the course of a few minutes with the assistance of sonication. To a PCR tube containing a 2.0 µL aliquot of 2.5 µg/µL ubiquitin in Milli-Q H_2O was added 18.0 µL of the freshly prepared P-imidazolide solution. The resulting clear, colorless solution was incubated at 45 ˚C with 400 rpm shaking for the desired reaction time. Ub-S65pS was found to be consumed by reagent **1** to yield primarily Ub-S65pS-PO_2HBn after 6 hours. The reaction solution was quenched 5x into an 80 µL aliquot of aqueous 0.1 M EDTA (pH 8.5) and then transferred to a 3 kDa MWCO centrifuge filter unit with a capacity of 500 µL. The sample was subject to four cycles of centrifugation (14,000 rcf, 20 min, 17 ˚C), diluting to 500 µL with 1x PBS buffer between spins. After the last spin, the protein aliquot was transferred to a tared, clean sample tube to estimate the final volume by weight (assuming a density of 1mg/µL). Samples were typically 27-40 µL.

Myo-D127pS Pyrophosphorylation in Aqueous Conditions:

To a a PCR tube containing a 2.0 µL aliquot of 2.5 µg/µL Myo-D127pS in Milli-Q H_2O was added 10 µL of 68 mM $ZnCl_2$ in Milli-Q H_2O, 5 µL of 40 mM reagent **1** or **3** in Milli-Q H_2O and 3 µl Milli-Q H_2O. The resulting clear, colorless solution was incubated at 37 ˚C with 500 rpm shaking for the desired reaction time. The reaction solution was quenched 5x into an 80 µL aliquot of aqueous 0.1 M EDTA (pH 8.5) and then transferred to a 3 kDa MWCO centrifuge filter unit with a capacity of 500 µL. The sample was subject to four cycles of centrifugation (15,000 rcf, 20 min, 17 ˚C), diluting to 500 µL with 1x PBS buffer between spins. After the last spin, the protein aliquot was transferred to a tared, clean sample tube to estimate the final volume by weight (assuming a density of 1mg/µL). Samples were typically 29-40 µL.

Circular Dichorism (CD) Spectroscopy:

CD spectra were obtained on a Jasco J-720 spectropolarimeter using Jasco J-700 series control driver software, version 1.08.00 [Build 3]. Data was analyzed with Jasco Spectra Analysis software, version 1.53.04 [Build 1]. Spectra were taken in a Hellma 100-QS Quartz SUPRASIL® Cuvette with a 1.0 mm path length. Samples were prepared by exchanging into Phosphate-Buffered Fluoride (PBF) buffer (consisting of 154 mM NaF, and 10 mM Na_2HPO_4 in Milli-Q H_2O adjusted to pH 7.4, made with 99.99% NaF (trace metals basis, from Sigma-Aldrich)) by at least five cycles of spin desalting (14,000 rcf, 20 min, rt) in 500 µL 10 kDa MWCO centrifugal filters, by diluting the sample to 500 µL with PBF between spins. Sample protein concentrations were adjusted to 13 µM as determined by UV absorbance at 280 nm. Sample volumes were at least 200 µL.

XI. Abbreviations

2,2'-DTDP – 2,2'-Dithiodipyridine or 2,2'-Dipyridyl disulfide
ACN – Acetonitrile
βME – β-mercaptoethanol
CHA – Cyclohexylamine
DCM – Dichloromethane
DiPEA – Diisopropylethylamine (Hünig's base)
DMA – *N*,*N*-Dimethylacetamide
DMF – *N*,*N*-Dimethylformamide
DMSO – Dimethylsulfoxide
EDTA – Ethylenediamenetetraacetic acid
FA – Formic acid

fc – flash chromatography
FDR – False Discovery Rate
FT – Fourier Transform
2,2'-DTDP – 2,2'-dithiodipyridine
HATU – 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
Im – Imidazole
MeLi – Methyl lithium
MES – 4-Morpholineethanesulfonic acid (buffer)
MOPS – 4-Morpholinepropanesulfonic acid (buffer)
MWCO – Molecular weight cutoff
Myo – Myoglobin
NiNTA – Nickel (II) nitriloacetic acid
NMA – *N*-Methylacetamide
NPE – Nitrophenylethyl
PBS – Phosphate-buffered saline
PEG – Polyethylene glycol
PPh_3 – Triphenylphosphine
PSM – Peptide Spectrum Match
rt – room temperature
TEA – Triethylamine
TFA – Trifluoroacetic acid
THPTA – Tris(3-hydroxypropyltriazolylmethyl)amine
TRIS – Tris(hydroxymethyl)aminomethane
Ub – Ubiquitin

XII. References

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