Lanthanide-mediated phosphoester hydrolysis and phosphate elimination from phosphopeptides

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~ Supplementary Information ~

I. Reaction conditions, materials, and peptide synthesis

Unless otherwise noted, all peptide immobilizations, washes, and kinetic assays were conducted at RT in "**BTP-NT"** buffer that contained 50 mM bis-tris propane • HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20.

Metals ions were obtained in 99.9% or higher purity from Sigma-Aldrich or Acros, and prepared in deionized water at 100 mM and stored in borosilicate glass at RT. The salts used were: Gd•Cl_3•6(H_2O), Eu•Cl_3•6(H_2O), Nd•Cl_3, Sm•Cl_3, La•Cl_3•7(H_2O), Tm•Cl_3•5(H_2O), Dy•(NO_3)$_3$•5(H_2O), Ce•(NO_3)$_3$•6(H_2O), Ce•(NH_4)$_2$•(NO_3)$_6$, Lu•Cl_3•6(H_2O), Pr•(NO_3)$_3$•5(H_2O), Er•(NO_3)$_3$•(H_2O), Tb•Cl_3•6(H_2O), Ho•Cl_3•6(H_2O), Yb•Cl_3•6(H_2O), Y•Cl_3•5(H_2O), Sc•Cl_3•5(H_2O), Cu•Cl_2•2(H_2O), Zn•(OAc)$_2$. Bis-tris propane was obtained from Sigma-Aldrich (>99% pure). Kinases were obtained from the following sources and were used without dilution: p60src kinase (Upstate, 75U/25 µL), PKA catalytic subunit (Promega, 2 mg/mL), rhcdc2 kinase (Promega 5U/µL), PKC [θ] (Upstate, 2U/55 µL), CaM II calmodulin-dependent kinase α-subunit (Calbiochem, 5000U/vial). 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine], and 1,2-dioleoyl-sn-glycerol were obtained from Avanti Polar Lipids. Streptavidin-coated agarose beads were obtained from Novagen. Cytidylyl(3'→5')guanosine ammonium salt (**CpG**), and 2' deoxycytidylyl(3'→5')-2'-deoxyguanosine ammonium salt (**dCpdG**) were obtained from Sigma-Aldrich.

Biotinylated peptides **P1 – P6** were obtained from Promega as part of the appropriate "SignTECT" kit. The biotinylated peptide **P7** was obtained from Anaspec (San Jose, CA). To provide non-radioactive, phosphorylated material for solution-phase control experiments, the biotinylated peptide **P3** was phosphorylated using the catalytic subunit of cAMP-dependent protein kinase (PKA) as described in Section II A except that no γ-^{32}P ATP was included in the reaction, and the final concentrations of ATP and **P3** were 500 µM and 100 µM, respectively. The reaction was incubated at RT for 1 hr, and the reaction components were separated using a Rainin Dynamax HPLC and a Vydac semipreparative column (C18, 300 Å, 10 µm, 10 mm x 250 mm) with a flow rate of 5 mL/min, and a 10 – 20% gradient of CH_3CN in H_2O (containing 0.1% TFA) from 1 – 20 min. A single new peak (retention time = 17 min) was collected, lyophilized, and mass evaluated using an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA). As expected, the mass of the product (m/z = 1,080) was 80 amu heavier than the starting material (m/z = 1,000). The phosphopeptide **P7** (lacking an N-terminal biotin) was obtained from Anaspec

(San Jose, CA). **P8 – P12** were synthesized with a Symphony® multi-channel solid-phase synthesis apparatus (Protein Technologies, Inc.) on a 25 μmole scale using standard Fmoc chemistry and Rink amide resin**.** The protected phosphoaminoacids used in the synthesis (Fmoc-Tyr (PO_3H_2)-OH, Fmoc-Ser (PO(OBzl)OH)-OH, and Fmoc-Thr (PO(OBzl) OH)-OH were obtained from Novabiochem (San Diego, CA). Following automated synthesis, peptides were simultaneously cleaved from the resin and deprotected for 2 hr at RT using 95% TFA (containing 1.7% of each: water, phenol, and triisopropyl silane). The peptides were then precipitated by the addition of 40 mL of diethyl ether. The precipitate was washed 1x with 40 mL of diethyl ether, dried under vacuum, and purified using a Rainin Dynamax HPLC and a Vydac semipreparative column (C18, 300 Å, 10 μm, 10 mm x 250 mm). A 2 – 20% gradient of CH_3CN in H_2O (containing 0.1% TFA) from 1 – 25 min and a flow rate of 5 mL/min were used. The major product from each synthesis was collected, lyophilized, and mass evaluated using a Waters Micromass ESI mass spectrometer. MS calculated for **P8**, $C_{24}H_{29}N_6O_8P$: 560, found 559 $[M]^{-1}$. MS calculated for **P9**, $C_{18}H_{25}N_6O_8P$: 484, found 483 $[M]^{-1}$. MS calculated for **P10**, $C_{19}H_{27}N_6O_8P$: 498, found 497 $[M]^{-1}$. MS calculated for **P11**, $C_{30}H_{37}N_6O_{12}P$: 704, found 703 $[M]^{-1}$. MS calculated for **P12**, $C_{28}H_{33}N_6O_{12}P$: 676, found 675 $[M]^{-1}$.

II. Detailed description of ^{32}P-release assay

A. Phosphorylation of peptides P1 – P7. ^{32}P-phosphorylation reactions were conducted using the biotinylated peptides and buffers provided in Promega's "SignaTECT" kinase assay kits for protein tyrosine kinase (PTK), cAMP-dependent protein kinase (PKA), protein kinase C (PKC), cdc2 protein kinase, and calcium/calmodulin-dependent protein kinase (CaM KII). A summary of the reaction conditions employed for each peptide is shown in Table S1. Each reaction was mixed in a 1.5 mL polypropylene tube in the following order: water, buffer, peptide, ATP/γ-^{32}P ATP mixture, and enzyme (undiluted). The γ-^{32}P "ATP mixture" was prepared by mixing water (152 μL), 0.5 mM cold ATP (150 μL), and 400 μCi of γ-^{32}P ATP (78 μL), to give a total ATP concentration of 200 μM. Given the specific activity and age of the γ-^{32}P ATP used (6000 Ci/mmol ATP), the final ratio of hot to cold ATP was 1:1200. The γ-^{32}P ATP mixture was added to each reaction so that final ratio of peptide to ATP was 2:1 for each reaction. All reactions were incubated for 1 hr at RT and carried directly over into the next step.

Table S1. Summary of phosphorylation reactions. All reactions incubated for 1 hr at RT.[a]

Peptide #	H_2O	SignTECT 5X buffer / and type	5X activation buffer	Peptide conc. And volume Added	Final Peptide Concentration	ATP Mix[d]	Enzyme Volume/ type
P1	7.5 µL	25 µL / PTK	-	12.5 µL x 2.5 mM	250 µM	80 µL	5µL p60src
P2	7.5 µL	25 µL / PTK	-	12.5 µL x 2.5 mM	250 µM	80 µL	5µL p60src
P3	43 µL	25 µL / PKA	-	25 µL x 0.5 mM	100 µM	32 µL	5µL PKA cat SU
P4	19 µL	25 µL / cdc2	-	50 µL x 0.25 mM	100 µM	32 µL	5µL rhcdc2
P5	19 µL	25 µL / PKC	25 µL[b]	25 µL x 0.5 mM	100 µM	32 µL	5µL PKC θ
P6	19 µL	25 µL / CaM	25 µL[c]	25 µL x 0.5 mM	100 µM	32 µL	5µL CaM
P7	1.5 µL	25 µL / PTK	-	21 µL x 1.5 mM	250 µM	80 µL	5µL p60src

[a] 1 µL of 10 mg/mL BSA was added to each reaction to give a final concentration of 0.08 mg/mL.
[b] Contains 100 mM bis-tris propane pH 8, 50 mM $MgCl_2$, 64 µg/mL 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine], and 4.8 µg/mL 1,2-dioleoyl-sn-glycerol. Activation buffer stored at -80 °C.
[c] Contains 5 mM $Ca(OAc)_2$, 5 µM calmodulin, 0.1 mg/mL BSA. Activation buffer stored at -20 °C.
[d] See text for preparation and activity.

B. Normalization for ^{32}P incorporation. Phosphorylation efficiencies were determined by taking a small aliquot of each reaction (Section II A), diluting it into "BTP-NT" buffer (Section I), incubating it with an excess of streptavidin-containing agarose beads, and using scintillation counting to determine the fraction of radioactivity in solution versus that associated with the beads. The final peptide and ATP concentrations in each crude phosphorylation reaction are summarized in Table S2. A small aliquot of each reaction was diluted into 500 µL with BTP-NT buffer for a final peptide concentration of 4.25 µM (Table S2). The diluted "crude" phosphorylation reactions were then added to 125 µL of pre-washed streptavidin-conjugated agarose beads and incubated for 1 hr at RT with constant inversion of each tube for mixing. The biotin binding-capacity of the agarose beads used is ≥85 nmoles/mL. This represents at least a 5-fold excess in biotin binding capacity as compared to moles of biotinylated peptide in each reaction. Peptide capture, therefore, was assumed to be quantitative. After centrifuging the samples at 1,000 rpm for 30 sec to settle the beads 10 µL (1.33 %) of the supernatant from each reaction was added to 10 mL of Ultima Gold™ liquid scintillation cocktail and counted for 1 min using a Packard 1500 Tri-Carb liquid scintillation analyzer (results summarized in Table S2). The remaining supernatant in each mixture was then discarded and the beads were washed 10 times with 0.5 mL of BTP-NT buffer (by adding the buffer, mixing each tube by inversion for 1 min, centrifuging the slurry at 1,000 rpm to settle the beads, removing the supernatant with a pipette and repeating). The radioactivity contained in 2.5 µL (1.0 %) of the washed beads was determined using liquid scintillation counting as described above. The % of ^{32}P incorporated into the agarose beads (versus the supernatant) was used to calculate the yield for each phosphorylation reaction (Table S2). Since a 2:1 ratio of peptide to ATP was used for

each reaction, the % of ^{32}P incorporated onto the solid-phase multiplied by 0.5 is equal to the percentage of peptide phosphorylated (Table S2). This calculation assumes that ATP and γ-^{32}P ATP are incorporated into each peptide with equal efficiencies. The phosphorylation efficiencies for **P1** – **P7** ranged between 1.7 % – 44.0 % (Table S2). To normalize the ratio of phosphorylated to nonphosphorylated peptide in each reaction mixture, non-phosphorylated (cold) peptide was added so that 1.7% of the peptide in each resulting mixture was phosphorylated (summarized in Table S3). An appropriate amount of the corresponding non-phosphorylated peptide and BTP-NT buffer was added to each reaction so that the final mixture contained 0.34 μM of phosphorylated peptide and 19.7 μM of the corresponding non-phosphorylated peptide (summarized in Table S3).

Table S2. Test immobilizations for determination of phosphorylation efficiencies.

Rxn #	Final Peptide Conc.	Final ATP Conc.	Vol. of Rxn. Added	Vol. Of BTP-NT Buffer Added	Vol. of Beaded Agarose Added[a]	^{32}P CPM in 1.33% of Supernatant	^{32}P CPM in 1% of Washed Beads	% of ^{32}P Incor-porated	% of Phospho-peptide in mix
P1	250 μM	125 μM	17 μL	483 μL	250 μL	226,424	33,198	17	8.7
P2	250 μM	125 μM	17 μL	483 μL	250 μL	156,330	75,261	41	20.4
P3	100 μM	50 μM	42.5 μL	458 μL	250 μL	31,007	160,588	88	44.0
P4	100 μM	50 μM	42.5 μL	458 μL	250 μL	239,339	9,443	5.3	2.7
P5	100 μM	50 μM	42.5 μL	458 μL	250 μL	50,786	144,514	80	40
P6	100 μM	50 μM	42.5 μL	458 μL	250 μL	187,462	53,920	29	14.6
P7	250 μM	125 μM	17 μL	483 μL	250 μL	273,112	6,532	3.3	1.7

[a] A 50% slurry (volume of beads / total volume) added.

Table S3. Normalization of phosphorylation reactions.

Rxn #	Final Peptide Conc.	% Phospho-peptide (Table S2)	Crude Rxn Vol Added [a]	Vol 7M Guand-HCl Added	Cold Peptide: Stock Conc. and Volume Added [b]	BTP-NT Buffer Added
P1	250 μM	8.7	7.8 μL	7.8 μL	3.2 μL x 2.5 mM	481 μL
P2	250 μM	20.4	3.3 μL	3.3 μL	3.7 μL x 2.5 mM	490 μL
P3	100 μM	44.0	3.9 μL	3.9 μL	19.2 μL x 0.5 mM	473 μL
P4	100 μM	2.7	63 μL	63 μL	14.8 μL x 0.25 mM	360 μL
P5	100 μM	40	4.2 μL	4.2 μL	19.2 μL x 0.5 mM	472 μL
P6	100 μM	14.6	11.6 μL	11.6 μL	17.7 μL x 0.5 mM	460 μL
P7	250 μM	1.7	40 μL	40 μL	-	420 μL

[a] Sufficient volume added so that 1.7×10^{-10} moles of phosphopeptide are included in each.
[b] Sufficient volume added so that 1×10^{-8} moles (total) of peptide are included in each.

C. Immobilization of P1–P7 onto streptavidin-coated agarose beads. 710 µL of a 50% slurry of streptavidin-coated agarose beads (355 µL of beads with a biotin binding capacity of ≥85 nmoles/mL) were washed three times with 500 µL of BTP-NT buffer by centrifuging the slurry at 1,000 rpm to settle the beads, then removing the supernatant with a pipette, adding fresh buffer, and mixing the tube by inversion. After the last wash, BTP-NT buffer was added so that the final volume was 710 µL (a 50% slurry). Each of the normalized phosphopeptide mixtures from Sections IIA–B (containing a total of 10 nmoles of each peptide with 1.7% phosphopeptide in 500 µL of buffer) were then added to the washed beads and incubated at RT with constant inversion of each tube for 1 hr. The beads from each reaction were then washed 10x with 700 µL of BTP-NT buffer as described above. Following the last wash, 30 mL of BTP-NT was added to the beads from each reaction to give a 1.2% slurry.

D. ^{32}P release in the presence of 21 metal ions. ^{32}P displacement assays were conducted by mixing a 400 µL aliquot of the 1.2 % slurry of the agarose-immobilized peptide (containing 1.1 x 10^{-11} moles of phosphopeptide) with 400 µL of BTP-NT buffer in a 1.7 mL microcentrifuge tube. To this 0.6 % slurry, 0.8 µL – 8 µL of a 100 mM metal ion stock solution was added (all metal ion stocks were aged 100 d in deionized water prior to use). The reaction was immediately mixed by inverting the tube and incubated at RT with constant inversion for mixing. Following the indicated incubation time, the slurry was centrifuged at 1,000 rpm for 1 min to settle the beads. A 500 µL aliquot of the supernatant was then added to 10 mL of Ultima Gold™ liquid scintillation cocktail and counted for 1 min using a Packard 1500 Tri-Carb liquid scintillation analyzer. Control experiments were performed to determine the minimum and maximum amount of ^{32}P that could be released from the beads. To determine the minimum amount of ^{32}P release from each peptide, analogous experiments were performed in the absence of any added metal ions. To determine the maximum amount of ^{32}P release, we added 500 µL of the 0.6 % slurry (rather than 500 µL of supernatant) to 10 mL of scintillation fluid and counted for ^{32}P as described above. The % of ^{32}P released from each immobilized peptide was established by the fractional displacement of ^{32}P in the presence of each metal ion relative to these controls. The % of ^{32}P released for each sample was then multiplied by the total moles of phosphopeptide present (1.1 x 10^{-11}) to determine the moles of phosphate released (Table S4).

Table S4. Moles of phosphate released ($x10^{12}$) from solid-phase immobilized peptides after 18 h in the presence of 1 mM of each metal ion.[a]

Ion	Peptide **P1**	Peptide **P2**	Peptide **P3**	Peptide **P4**	Peptide **P5**	Peptide **P6**	Peptide **P7**
None	0.00	0.00	0.00	0.01	0.02	0.02	0.00
Mg(II)	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Ca(II)	0.00	0.01	0.01	0.01	0.00	0.01	0.01
Sc(III)	0.00	0.01	0.02	0.00	0.02	0.00	0.01
Cu(II)	0.00	0.00	0.02	0.01	0.01	0.03	0.06
Zn(II)	0.00	0.00	0.02	0.01	0.00	0.00	0.00
Y(III)	0.11	0.07	0.09	0.02	0.06	0.00	0.12
La(III)	0.57	0.56	0.19	0.00	0.01	0.02	0.14
Ce(III)	0.76	0.91	0.29	0.00	0.12	0.12	1.18
Ce(IV)	2.78	1.99	0.06	0.02	0.10	0.18	3.06
Pr(III)	0.12	0.13	0.04	0.00	0.02	0.10	0.17
Nd(III)	0.20	0.19	0.09	0.00	0.00	0.10	0.15
Sm(III)	0.14	0.12	0.08	0.00	0.00	0.02	0.19
Eu(III)	0.10	0.09	0.08	0.00	0.02	0.01	0.13
Gd(III)	0.11	0.09	0.10	0.00	0.03	0.01	0.22
Tb(III)	0.02	0.06	0.06	0.00	0.03	0.00	0.06
Dy(III)	0.08	0.04	0.06	0.00	0.02	0.00	0.12
Ho(III)	0.04	0.03	0.02	0.00	0.02	0.01	0.07
Er(III)	0.01	0.04	0.06	0.00	0.04	0.02	0.07
Tm(III)	0.04	0.04	0.07	0.00	0.00	0.00	0.13
Yb(III)	0.07	0.07	0.03	0.02	0.01	0.00	0.00
Lu(III)	0.09	0.06	0.03	0.02	0.01	0.00	0.07

[a] Estimated standard deviations are less than or equal to +/-30% of each reported value.

III. Product analyses of P3, P7, and P8 – P12 by HPLC and mass spectrometry

A. Dephosphorylation of P3 and P7 by La(III) and Ce(IV). The biotinylated phosphopeptide **P3** (50 μM in 50 mM bis-tris propane (pH 8.0)) was treated with 1 mM of Ce(IV) or 10 mM La(III) for 24 hrs at 37 ^{o}C and desalted using a Water's C-18 reversed phase Sep-Pak. Mock reactions with no Ce(IV) or La(III) were also prepared. MALDI TOF MS analysis was conducted with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA) using a-cyano-4-hydroxycinnamic acid as the ionization matrix. A new peak for the Ce(IV)-treated sample with a change in m/z = -80 amu relative to the control sample is revealed (Figure S1 A, B). For the La(III)-treated sample, a new peak with a change in m/z = -98 amu relative to the control sample is observed (Figure S1 A, C). These results are consistent with Ce(IV)-meidated phosphoester hydrolysis of **P3**, and La(III)-mediated phosphate elimination of **P3**.

50 μM of the phosphopeptide **P7** (lacking an N-terminal biotin) was treated with 1 mM of Ce(IV) or 1 mM of La(III) in BTP-NT buffer at RT or 37 ^{o}C for 30 min or 2 hrs. Mock reactions with no Ce(IV) or La(III) were also prepared. Each reaction was desalted and analyzed by MALDI TOF MS as above. Analysis of **P7** following either Ce(IV) or La(III) treatment shows the formation of a single product with a mass of -80 amu relative to control samples (Figure S1 D–F).

B. Dephosphorylation analysis of P3 by HPLC. The biotinylated phosphopeptide **P3** (50 μM in 50 mM bis-tris propane (pH 8.0)) was treated with 1 mM Ce(IV) at 37 ^{o}C, and the reaction monitored by analytical HPLC. 20 μL aliquots of the mixture at various time points were injected onto a Vydac analytical column (C18, 300 Å, 5 μm, 4.6 mm x 150 mm) and separated using a flow rate of 1.0 mL/min, and a 20 – 30% gradient of CH_3CN in H_2O (containing 0.1% TFA) from 1 – 20 min. The elution of products was monitored at 215 nm. An overlay of these chromatographs (Figure S2) shows that with increasing time, the peak that corresponds to the starting material disappears (RT = 12 min), while a new peak with a slightly longer retention time grows in (RT = 13 min). This new product has an identical retention time as a synthesized standard of non-phosphorylated P3, and its mass is consistent with the hydrolysis of the phosphate from phosphopeptide P3 (change in m/z = -80 amu = HPO_3). Unlike **P3**, the phosphorylated versus non-phosphorylated species of **P7**, could not be resolved by HPLC, making accurate quantification of product formation kinetics difficult.

For this reason, a series of short, homologous, phosphopeptides **P8 – P12** were synthesized and used for detailed kinetic studies (below).

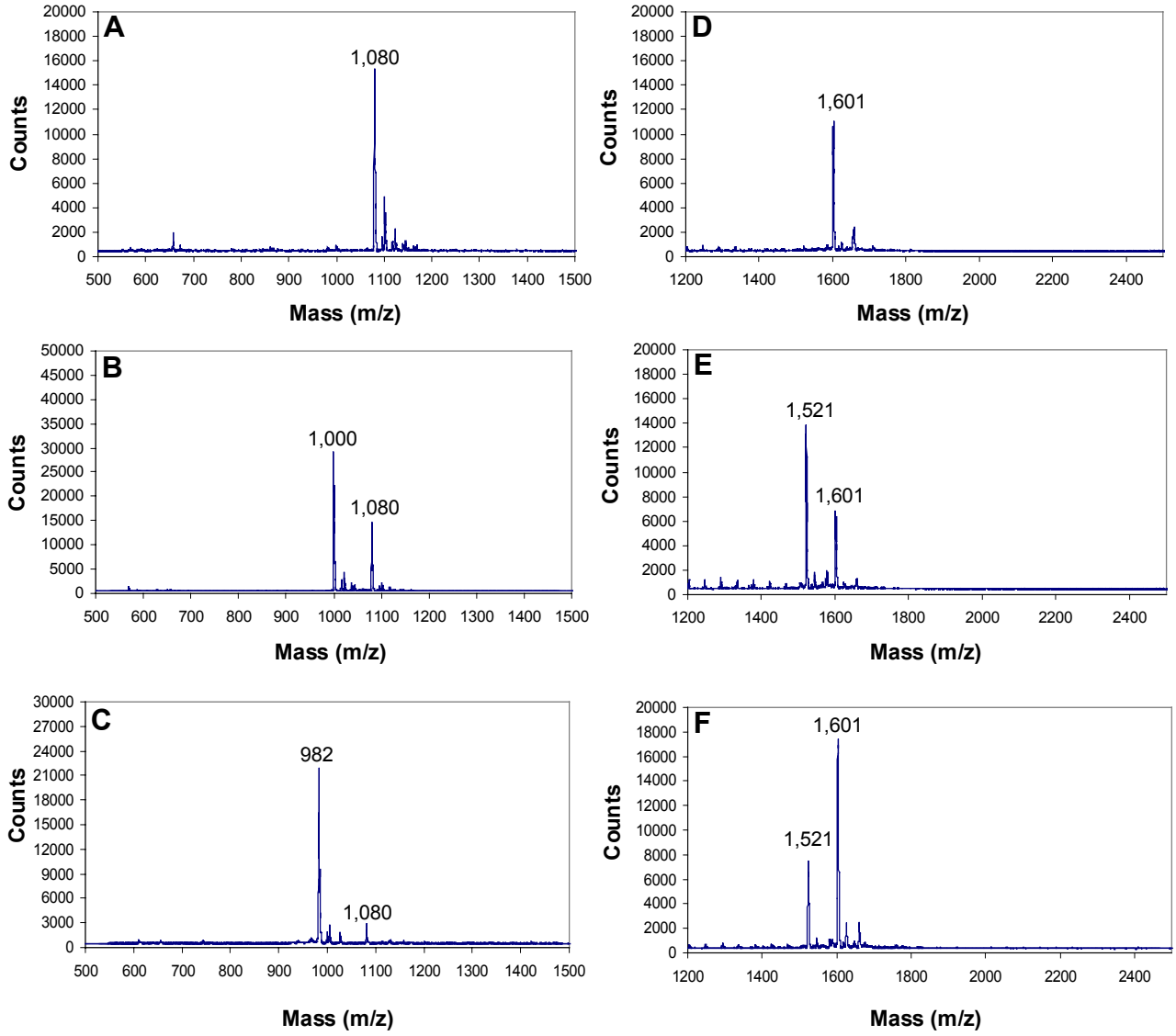

Figure S1. MALDI TOF MS analysis of phosphopeptide **P3** and **P7** upon treatment with Ce(IV) or La(III). (**A**) Mock reaction containing **P3** and buffer after a 24 hr incubation at 37 ^{o}C. (**B**) A mixture of phosphopeptide **P3** and 1 mM Ce(IV) in 50 mM bis-tris propane (pH 8.0) after a 24 hr incubation at 37 ^{o}C. (**C**) A mixture of phosphopeptide **P3** and 10 mM La(III) after a 24 hr incubation at 37 ^{o}C. (**D**) Mock reaction containing **P7** and BTP-NT buffer after a 2 hr incubation at 37 ^{o}C. (**E**) A mixture of phosphopeptide **P7** and 1 mM Ce(IV) in BTP-NT buffer following a 30 min incubation at 25 ^{o}C. (**F**) A mixture of phosphopeptide **P7** and 1 mM La(III) after a 2 hr incubation at 37 ^{o}C.

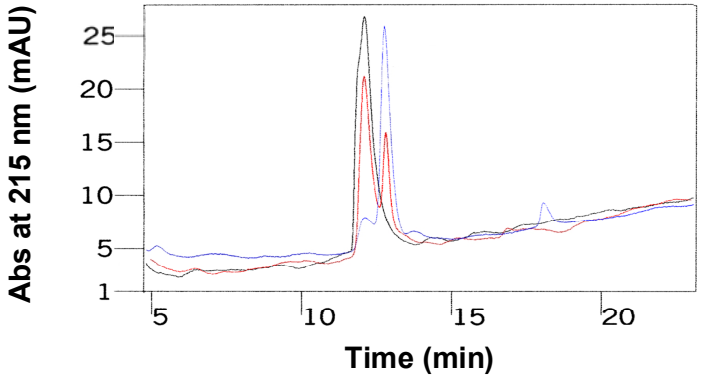

Figure S2. HPLC chromatographs of a reaction containing the phosphorylated, biotinylated peptide **P3** and 1 mM Ce(IV) at three different time points. Aliquots of the same reaction mixture were injected at t = 0 hrs (black chromatograph), t = 17 hrs (red chromatograph), and t = 48 hrs (blue chromatograph). The peaks at 12 and 13 min were collected, concentrated and analyzed by MALDI TOF MS. The peak at 12 min has a retention time and mass consistent with the starting material (m/z = 1,080). The peak at 13 min has a retention time and mass consistent with the dephosphorylated peptide **P3** (m/z = 1,000). Mock reactions that lack Ce(IV) show no product formation after 72 hrs (same as black chromatograph).

C. Ce(IV)-mediated dephosphorylation of P8 – P12. All reactions were conducted by adding 10 µL of a 100 mM metal ion solution into 990 µL of 10 µM phosphopeptide in BTP-NT buffer. Reactions were incubated at RT and at various time points (ranging from 1 min – 220 hours), an aliquot of the reaction (190 µL) was stopped by mixing it with 10 µL of a solution containing 250 mM sodium EDTA and 100 mM sodium phosphate (pH 7.4). The quenched reactions were stored at -20 ^{o}C until 100 µL of each reaction mixture was analyzed using a Waters HPLC equipped with a Vydac analytical column (C18, 300 Å, 5 µm, 4.6 mm x 150 mm) and a 5 – 20% gradient of CH_3CN in H_2O (containing 0.05% TFA) from 1 – 25 min with a flow rate of 1.0 mL/min. The elution of products was monitored at 260 nm (figure S3). Peaks corresponding to the starting material and products were collected, concentrated and analyzed by MALDI TOF MS.

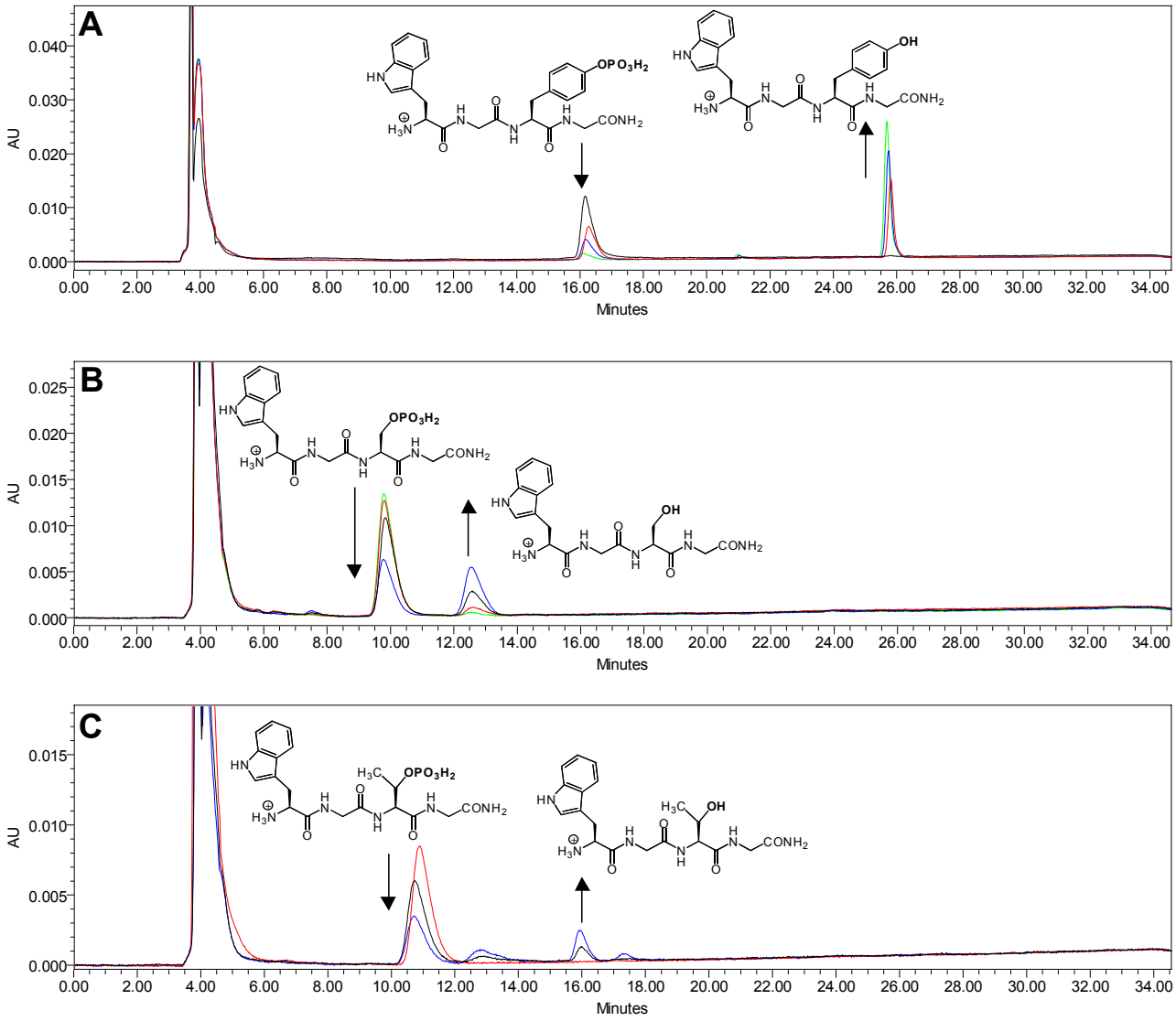

Figure S3. HPLC chromatographs illustrating Ce(IV)-mediated phosphoester hydrolysis. Arrows indicate the change in intensity of each peak with increasing reaction time. Product identities were confirmed by mass spectrometry and by co-injection with authentic samples. (**A**) **P8** and 1 mM of Ce(IV) at 0 min (black), 2 min (red), 6 min (blue), and 18 min (green) incubation times. Nearly identical results are obtained for **P11 – P12** as for **P8** (not shown). (**B**) **P9** and 1 mM Ce(IV) after 6 min (green), 22 min (red), 10 hrs (black), and 24 hrs (blue). (**C**) **P10** and 1 mM Ce(IV) after 22 min (red), 24 hrs (black), and 76 hrs (blue). The two side-products observed in (**C**) have masses consistent with the conversion of the C-terminal amide of **P10** into a C-terminal acid, resulting in a slightly longer retention time for both the starting material and product.

D. Ce(IV)- and La(III)-mediated dephosphorylation of P9. 10 µL of a 100 mM Ce(IV) or La(III) stock solution (prepared in deoinized water) was added to a solution containing 990 µL of a 10 µM

solution of **P9** in BTP-NT buffer. Reactions were incubated at RT and at various time points, an aliquot of the reaction (190 µL) was stopped by adding 10 µL of a solution containing 250 mM sodium EDTA and 100 mM sodium phosphate (pH 7.4). The quenched reactions were stored at -20 ^{o}C until 100 µL of each reaction mixture was analyzed using a Waters HPLC equipped with a Vydac analytical column (C18, 300 Å, 5 µm, 4.6 mm x 150 mm) and a 5 – 20% gradient of CH_3CN in H_2O (containing 0.05% TFA) from 1 – 25 min with a flow rate of 1.0 mL/min. The elution of products was monitored at 260 nm (Figure S4). Peaks corresponding to the starting material and products were collected, concentrated and analyzed by MALDI TOF MS.

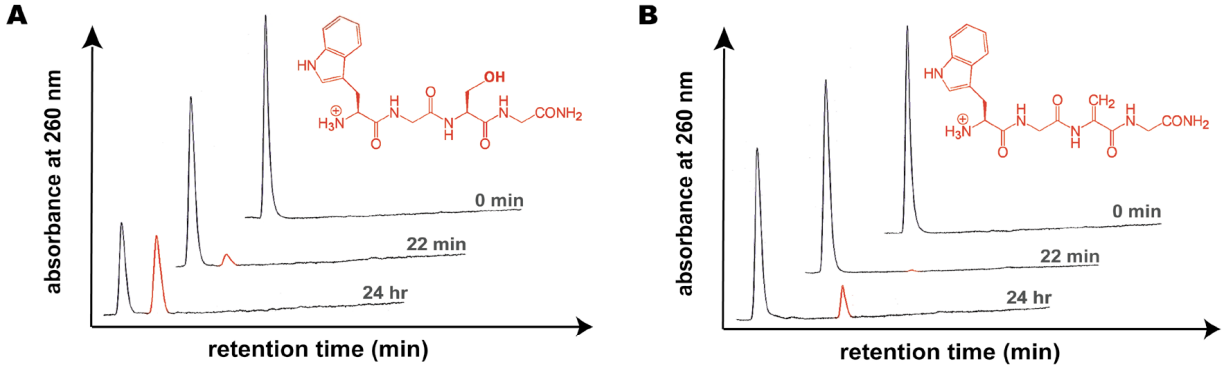

Figure S4. (**A**) HPLC analysis of a reaction containing **P9** (10 µM) and Ce(IV) (1 mM) after 0 min, 22 min, and 24 hours. (**B**) HPLC analysis of a reaction containing **P9** (10 µM) and La(III) (1 mM) after 0 min, 22 min, and 24 hrs (**B**). Product identities (shown in red) were confirmed by mass spectrometry and co-injection with an authentic sample. Mock reactions that lacked lanthanides showed no product formation after 2 weeks at RT.

E. Analysis of CpG and dCpdG cleavage. Cytidylyl(3'→5')guanosine ammonium salt (**CpG**), and 2'-deoxycytidylyl(3'→5')-2'-deoxyguanosine ammonium salt (**dCpdG**) were dissolved in deionized water to a final concentration of 5 mM. Each dinucleotide monophosphate (10 µM) was treated with 1 mM of Ce(IV) in BTP-NT buffer at 25 ^{o}C. At various time points (ranging from 24 – 220 hours), an aliquot of the reaction (190 µL) was stopped by adding 10 µL of a solution containing 250 mM sodium EDTA and 100 mM sodium phosphate (pH 7.4). The reactions were then stored at -20 ^{o}C until 100 µL of each reaction mixture was analyzed on a Waters HPLC using a Vydac analytical column (C18, 300 Å, 5 µm, 4.6 mm x 150 mm). The reaction components were separated using a 2 – 10% gradient of CH_3CN in H_2O (containing 0.1% TFA) from 1 – 25 min with a flow rate of 1.0 mL/min. The elution of products was monitored at 260 nm.

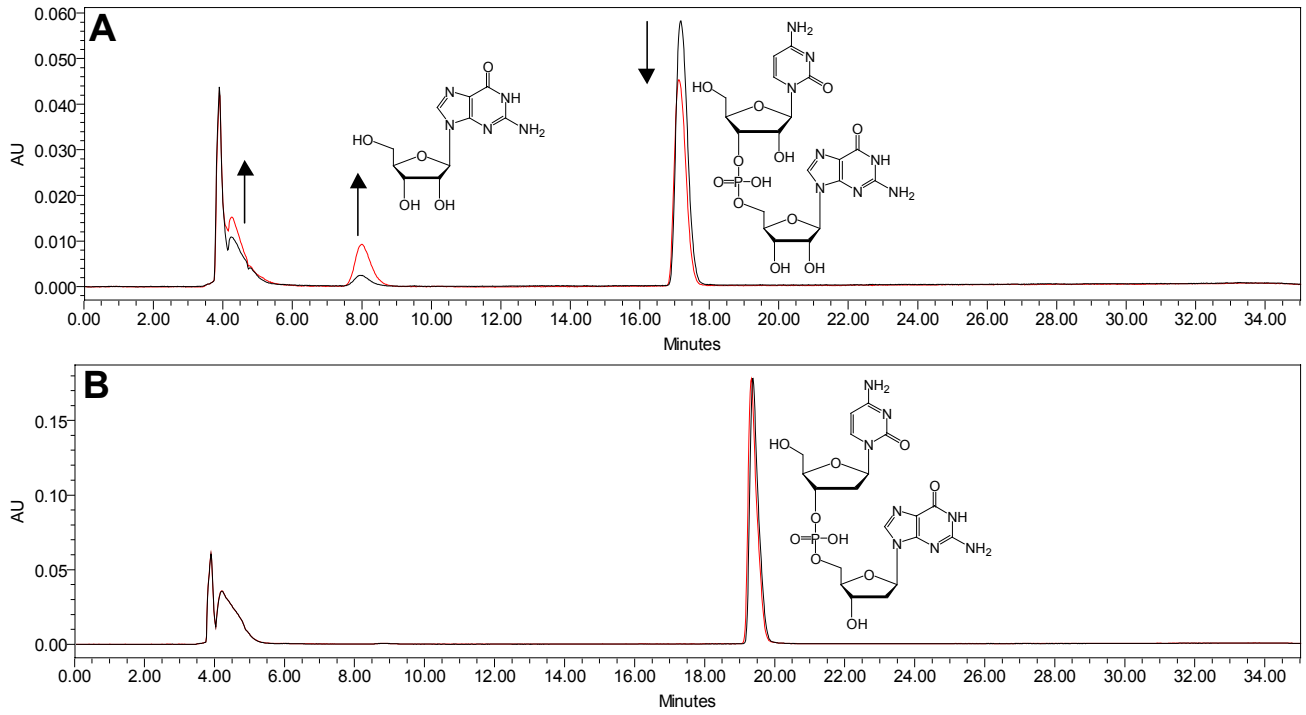

Figure S5. HPLC chromatographs showing a mixture of 1 mM Ce(IV) and either **CpG** (**A**) or **dCpdG** (**B**). Arrows indicate the change in intensity of each peak with increasing reaction time. (**A**) **CpG** at 24 hr (black) and 76 hr (red). (**B**) **dCpdG** at 24 hr (black) and 201 hr (red). Product identity (guanosine) was confirmed by mass spectrometry and co-injection with an authentic sample.

IV. Kinetic analysis of phosphate cleavage.

Reactions were prepared and analyzed as described in Sections IIIC-D. Rate constants have been determined from the initial rates of product formation according to integration of HPLC chromatographs. For **P8 – P12** the extinction coefficients (at 260 nm) of starting material and product are approximately the same, so the area of the product peak, divided by the total area (starting material peak + product peak) equals the fractional product conversion. This value multiplied by the total concentration of peptide equals the concentration of product at each time point. Initial reaction rates were determined by non-linear curve fit of these data to a hyperbolic curve where Y (concentration of product) = (maximum product concentration) * ((time (sec) / (half-life + time (sec)). Initial reaction rates were taken as the slope of the resulting curve over the first second of the reaction. Apparent second-order rate constants were calculated as k = (initial rate of product formation) / ([total concentration of metal ion]*[total concentration of substrate]). This

assumes that the reaction is first-order in both metal ion and substrate which has been confirmed for the Ce(IV)-mediated dephosphorylation of **P8** (Figure S6). The kinetic parameters for CpG cleavage have been determined in a similar fashion, except that differences in the extinction coefficients of the starting material and product were taken into account. The ratio of the extinction coefficients (at 260 nm) of CpG and the well-resolved product guanosine is approximately 1.5. The fractional conversion to product = (area of the product peak * 1.5) / ((area of the starting material) + (product peak x 1.5)). This value is multiplied by the total concentration of CpG to equal the concentration of product for each time point, and fit to an initial rate as described above.

B. Reaction orders for Ce(IV)-mediated dephosphorylation of P8

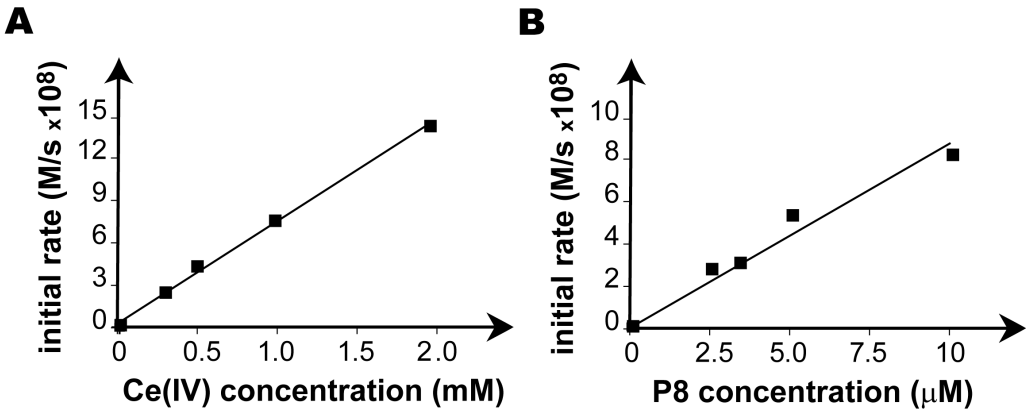

Figure S6. Initial reaction rates for **P8** dephosphorylation as a function of Ce(IV) concentration (**A**) and **P8** concentration (**B**). Reactions were conducted and analyzed as described in sections IIIC-D. Initial rates from three independent trials (conducted as described in section IIIC) were averaged and plotted above. For reactions conducted in parallel, error bars (standard deviations) were similar in size to the points on the graph. The aqueous Ce(IV) stock solutions used for these particular experiments were aged 60 d in deionized water before use. Similar apparent reaction orders were obtained with a fresh (2 hr old) stock of Ce(IV) (not shown). The apparent reaction order approaches zero-order in **P8** when the concentration of **P8** exceeds 20 μM. This may indicate saturated binding or at high substrate concentrations the activity of Ce(IV) species might be disrupted by aggregation or binding to multiple substrate molecules. Indeed, incomplete cleavage is observed as the concentration of **P8** approaches that of Ce(IV), and multiple turnover of substrate is not observed under these conditions (due perhaps to inhibition of Ce(IV) by precipitation with inorganic phosphate).

C. Dephosphorylation rates of P8 versus Ce(IV) stock solution age

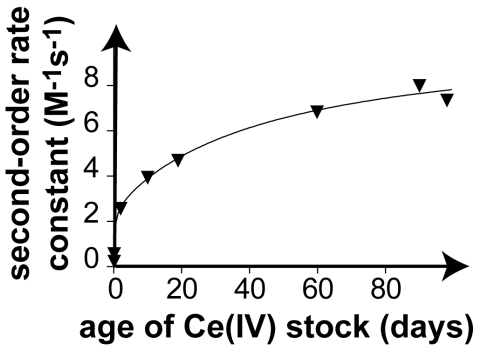

Figure S7. Apparent second-order rate constants for dephosphorylation of **P8** by Ce(IV) as a function of Ce(IV) stock solution age (reactions conducted and analyzed as described in sections IIIC-IV). Individual stock solutions (prepared at 100 mM with deionized water in borosilicate glass and stored at RT) were made over a 100 day period and evaluated side-by-side. For all reactions, a single product consistent with phosphate hydrolysis was observed. An activation similar to that observed at 100d was observed upon heating a fresh 100 mM stock of Ce(IV) in water at 90 °C for 10 min.