Supporting Information for

A General Method for the Synthesis of Caged Phosphopeptides: Tools for the Exploration of Signal Transduction Pathways

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Experimental:

All peptide synthesis reagents were purchased from Applied Biosystems or Novabiochem, and all other chemicals were purchased from Aldrich. ^{1}H NMR spectra were acquired on a Bruker Avance (DPX) 400 MHz spectrometer. ^{31}P NMR spectra were acquired on a Varian Mercury 300 MHz spectrometer, and all MAS ^{31}P NMR spectra were acquired on a Varian INOVA 500 MHz spectrometer. Chemical shifts are reported in ppm from a standard (tetramethylsilane for ^{1}H, H_2PO_4 for ^{31}P), and *J* values are in hertz. Thin-layer chromatography (TLC) was carried out on Merck 60 F_{254} 250-μm silica gel plates. High-performance liquid chromatography was performed using a Waters 600E HPLC fitted with a Waters 600 automated control module and a Waters 2487 dual wavelength absorbance detector recording at 228 and 280 nm. For analytical HPLC a Beckman Ultrasphere C_{18}, 5 μm, 4.6 x 150 mm reverse-phase column was used. For preparative separations a YMC-pack, C_{18}, 250 x 20 mm reversed phase column was used. The standard gradient for analytical and preparatory HPLC used was 93:7 to 0:100 over 35 minutes (water:acetonitrile, 0.1% TFA). Electrospray Ionization Mass Spectrometry (ESIMS) was performed on a PerSeptive Biosystems *Mariner™ Biospectrometry Workstation* (Turbo Ion Source).

1-(2-nitrophenyl)ethanol (NPE-OH)[16] To 2-nitroacetophenone (1.0 g, 6.05 mmol) in 14.4 mL of methanol:dioxane (3:2 by volume) sodium borohydride (687 mg, 18.17 mmol) was added and stirred in a cool water bath for 20 minutes. The mixture was allowed to stir for 2.5 hours longer at room temperature (progress monitored by TLC in chloroform). The reaction was quenched with 50 mL of water, stirring at room temperature for 30 minutes. The mixture was extracted into chloroform (3 x 30 mL), dried over sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. The alcohol was dried *in vacuo* over night to give a yellow oil, 990.9 mg (97.9%): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.9 (d, J= 8.0 Hz, 1 H), 7.9 (d, J= 8.0 Hz, 1 H), 7.7 (t, J= 7.2 Hz, 1 H), 7.4 (t, J= 7.6 Hz, 1 H), 5.4 (q, J= 6.4 Hz, 12.4 Hz, 1 H), 2.4 (s, 1H), 1.6 (d, J= 6.4 Hz, 3H).

O-*1-(2-nitrophenyl)ethyl*-O'-*β-cyanoethyl*-N,N-*diisopropylphosphoramidite (***1** *)*. A solution of 2-cyanoethyl diisopropylchlorophosphoramidite (**1**) (471 μL, 2.11 mmol) in 1.7 mL of anhydrous DCM was added to a stirring solution of nitrophenylethanol (294 mg, 1.76 mmol) and freshly distilled triethylamine (589 μL, 4.23 mmol) in 8.8 mL of anhydrous DCM at room temperature in the dark. The reaction was monitored by the disappearance of the nitrophenylethanol by TLC in 65:25:4 methanol:chloroform:water. The reaction mixture was washed with 10% sodium bicarbonate solution ($NaHCO_3$) (2 x 30 mL). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The product was dried *in vacuo* overnight to give a dark yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.9 (m, 2 H), 7.7 (m, 1 H), 7.4 (m, 1 H), 5.6 (m, 1 H), 3.9 (m, 2 H), 3.7 (m, 2 H), 2.5 (m, 2 H), 1.2 (m, 12 H), 1.6 (dm, J= 2.4 Hz, 6.8 Hz, 3H). ^{31}P NMR (121 MHz, $CDCl_3$) δ: 148.2 (d, J= 45.6 Hz).

General Peptide Synthesis Procedures. Peptides were prepared by standard Fmoc SPPS on PAL-PEG-PS resin. For example, for Fmoc-Ser-Pro-Gly (cpCS), 1.00 g (200 μmol) Fmoc-PAL-PEG-PS (0.2 mmole/g loading; 1% DVB) was swollen for five minutes with 5 mL of DCM followed by five minutes with 5 mL of DMF in a 100 mL peptide synthesis reaction vessel. The resin was deblocked with three five minute washes

of 5 mL of 20% piperidine in DMF, then rinsed five times for one minute each with 5 mL of DMF.

In each coupling step, 400 μmol of Fmoc-protected amino acid and 400 μmol of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) dissolved in 5.0 mL of DMF were added to the resin, and the coupling was initiated by addition of 800 μmol of diisopropylethylamine (DIEA). Each coupling was allowed to mix at room temperature for at least one hour. The resin was then rinsed two times for one minute with 5 mL of DMF and then two times for one minute with 5 mL of DCM. After each coupling reaction, a few resin beads were tested with trinitrobenzene sulfonic acid (TNBS) to check for free amines. Upon a negative test, the amino acid was deblocked as above for chain elongation. Residues to be caged were coupled using the corresponding amino acid with an unprotected hydroxyl side chain. Peptides were cleaved with 90% trifluoroacetic acid (TFA), 5% DCM, 2.5% water, and 2.5% triisopropylsilane (TIS) and triturated in ice-cold diethyl ether, unless otherwise noted. Concentrations of the final stock solutions were determined by quantitative amino acid analysis of hydrolyzed peptides (2 hours at 145°C with 6 N HCl and phenol), or by UV absorption of the nitrophenyl group (ε_{max} at A_{259} = 5700 $M^{-1}cm^{-1}$ in MeOH).

cpErk: Ac-PL-phospho(2-nitrophenylethyl)seryl-PAKLAFQFP-CONH$_2$

Fmoc-SPAKLAFQFP-(PAL-PEG-PS). Fmoc-SPAKLAFQFP-(PAL-PEG-PS) was synthesized using standard Fmoc-SPPS as described above. Data for a portion of cleaved resin: Analytical reversed phase HPLC, t_R= 26.2 min. MS (ESI), *m/z* calculated for $C_{69}H_{91}N_{13}O_{14}$: 1327.5 $(M+H)^+$ and 664.3 $(M+2H)^{2+}$. Found *m/z* 1326.8 $(M+H)^+$ and 663.9 $(M+2H)^{2+}$.

Fmoc-phosphi-(1-nitrophenylethyl-2-cyanoethyl)seryl-PAKLAFQFP-(PAL-PEG-PS). The resin-bound decapeptide Fmoc-SPAKLAFQFP-(PAL-PEG-PS) (200 mg resin, 40 μmol) was dried overnight *in vacuo*. A portion of **1** (73.5 mg, 200 μmol) was added the resin, and the vessel was flushed with nitrogen. The resin was swollen in 3.0 mL of solvent A (50:50 anhydrous DCM:anhydrous THF) for 10 minutes. The system was charged with 1*H*-tetrazole (14 mg, 200 μmol) and flushed again with nitrogen. The reaction was allowed to agitate overnight in the dark, upon which 3.0 mL of 10% $NaHCO_3$ solution was added to the reaction mixture and allowed to mix for several minutes. The resin was rinsed with solvent A (2 x 5 mL), and then further with DCM (2 x 5 mL).

Fmoc-phospho-(1-nitrophenylethyl-2-cyanoethyl)seryl-PAKLAFQFP-(PAL-PEG-PS). In a peptide synthesis vessel, Fmoc-phosphi-(1-nitrophenylethyl-2-cyanoethyl)seryl-PAKLAFQFP-(PAL-PEG-PS) (200mg resin, 40 μmol) was swollen in DCM. The resin was placed in 3.0 mL of DCM. A portion of *m*CPBA (13.8 mg, 80 μmol) was added to the solution and allowed to agitate for one hour at room temperature in the dark. The reaction mixture was then treated with 3.0 mL of 10% $NaHCO_3$ solution for 5 minutes, and the resin was rinsed with DCM (3 x 5 mL). Reversed phase HPLC revealed one major and one minor peak. The minor peak (t_R = 26.76 min, 26.5%) corresponded to the starting material. The major peak (t_R = 28.76 minutes, 73.5%) was the C-terminal desired product. MS (ESI), *m/z* calculated for $C_{80}H_{102}N_{15}O_{19}P$: 1609.7 $(M+H)^+$ and 805.4 $(M+2H)^{2+}$. Found *m/z* 805.0 $(M+2H)^{2+}$ and 816.0 $(M+H+Na)^{2+}$.

Ac-PL-phospho(nitrophenylethyl)seryl-PAKLAFQFP-CONH$_2$. Chain elongation was carried out by standard Fmoc SPPS in the dark. After the last residue was added, the *N*-terminus was acetyl capped with acetic anhydride (37.8 µL, 400 µmol) and pyridine (32.3 µL, 400 µmol). Reverse phase HPLC (t_R = 24.6 min). MS (ESI) *m/z* calculated for $C_{75}H_{109}N_{16}O_{20}P$: 1586.7 $(M+H)^+$ and 793.9 $(M+2H)^{2+}$. Found *m/z* 793.5 $(M+2H)^{2+}$, 804.5 $(M+H + Na)^{2+}$, and 815.5 $(M+2Na)^{2+}$.

cpChk2: Ac-MARHFD-phospho(nitrophenylethyl)-threonyl-YLIRR-CONH$_2$

Fmoc-TYLIRR-(PAL-PEG-PS). Fmoc-TYLIRR-(PAL-PEG-PS) was prepared using standard Fmoc SPPS as described above. Data for a portion of cleaved resin: Analytical reversed phase HPLC (t_R = 27.4 min). MS (ESI) *m/z* calculated for $C_{52}H_{75}N_{13}O_{10}$: 1042.3 $(M+H)^+$ and 522.1 $(M+2H)^{2+}$. Found *m/z* 521.8 $(M+2H)^{2+}$.

Fmoc-phospi-(1-nitrophenylethyl-2-cyanoethyl)-TYLIRR-CONH$_2$. Fmoc-TYLIRR-(PAL-PEG-PS) (114 mg resin, 25 µmole) was pumped dry *in vacuo* overnight. A portion of **2** (92 mg, 250 µmol) was added to the resin and the system was flushed with argon. The resin was swollen in anhydrous DCM (2.5 mL) for 15 minutes. The system was charged with 1*H*-tetrazole (17.5 mg, 250 µmole), then flushed again with argon. The reaction was stirred for 24 hours in the dark, after which 4.0 mL of saturated $NaHCO_3$ was added, and then the resin was rinsed with DCM (2 x 5mL).

Fmoc-phosho-(1-nitrophenylethyl-2-cyanoethyl)-threonyl-YLIRR-CONH$_2$. Fmoc-phospi-(1-nitrophenyl-ethyl-2-cyanoethyl)-TYLIRR-(PAL-PEG-PS) (114 mg resin, 25 µmol) was swollen with DCM in a peptide synthesis vessel. Then 2.8 mL of DCM and *m*CPBA (8.6 mg, 50 µmol) were added to the resin, and the solution was allowed to agitate in the dark for 1 hour. 6.0 mL of saturated $NaHCO_3$ was added to the swirling solution. After 5 minutes, the resin was rinsed with DCM (2 x 5 mL). The resin was cleaved in 95 %TFA, 2.5 % water, 2.5 % TIS, and triturated with diethyl ether. Analytical reversed phase HPLC revealed two peaks (t_R = 28.64 min, 28.82 min, no baseline separation). MS (ESI) *m/z* calculated for $C_{63}H_{85}N_{15}O_{15}P$: 1324.5 $(M+H)^+$ and 663.3 $(M+2H)^{2+}$. Found *m/z* 662.9 $(M+2H)^{2+}$, suggesting that they are diastereomers. The conversion to product, as determined by normalized areas on the HPLC trace, is 76%.

Ac-MARHFD-phospho(nitrophenylethyl)-threonyl-YLIRR-CONH$_2$. The peptide sequence was completed by standard Fmoc SPPS in the dark. The *N*-terminus was acetyl capped with acetic anhydride (26 µL, 0.28 mmole) and pyridine (23 µL, 0.28 mmole). The peptide was cleaved from the resin in 92.5 %TFA, 2.5 % water, 2.5 % TIS, 2.5 % EDT, and triturated with diethyl ether. Analytical reversed phase HPLC (t_R = 19.91 min, 83%). MS (ESI) *m/z* calculated for $C_{83}H_{124}N_{26}O_{22}PS$: 1848.0 $(M+H)^+$ and 617.0 $(M+2H)^{2+}$. Found *m/z* 617.0 $(M+2H)^{2+}$. The purified peptide was dissolved in deoxygenated water at pH 7.0 and stored at -20°C.

cpPax: Ac-EEEHV-phospho-(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosylSFPNKQK-CONH$_2$

Fmoc-YSFPNKQK-CONH$_2$. Fmoc-YSFPNKQK-(PAL-PEG-PS) was made using standard Fmoc SPPS as described above. Data for a portion of cleaved Fmoc-YSFPNKQK-$CONH_2$: Analytical reversed phase HPLC (t_R = 21.4 min). MS (ESI) *m/z* calculated for $C_{62}H_{81}N_{13}O_{14}$: 1231.60 $(M+H)^+$, 616.8 $(M+2H)^{2+}$. Found *m/z*: 617.0 $(M+2H)^{2+}$.

Fmoc-phosphi-(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosyl-SFPNKQK-CONH$_2$. Fmoc-YSFPNKQK-(PAL-PEG-PS) (230 mg resin, 37 μmol) was dried overnight under reduced pressure, and placed in an oven dried peptide synthesis vessel with molecular sieves, Type 4 Å, 8-12 mesh beads. The resin was swollen for 5 minutes with 3.0 mL of solvent A. In a separate round bottom flask, **1** (137 mg, 368 μmol) was mixed with 1*H*-tetrazole (26 mg, 368 μmol) in 2.0 mL of solvent A in the dark. The resulting mixture was added over the resin in solution. Argon was passed through the reaction mixture for 8 hours. The resin was washed with 5% aqueous $NaHCO_3$ (2 x 10 mL x 5 min), DMF (2 x 5 mL x 5 min) and DCM (2 x 5 mL x 5 min). The resin was separated from the molecular sieves by a pipet.

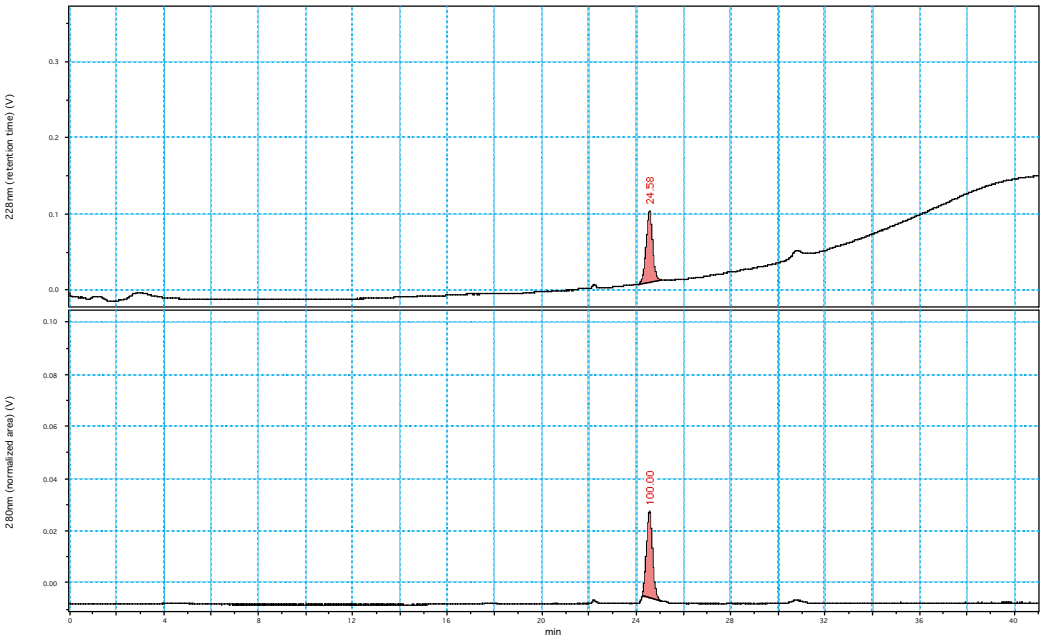
Fmoc-phospho-(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosyl-SFPNKQK-CONH$_2$. Fmoc-phosphi-(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosyl-SFPNKQK-(PAL-PEG-PS) (200 mg resin, 32 μmol) was dried overnight under reduced pressure and placed in an oven dried peptide synthesis flask. The resin was swollen with 4.0 mL of dry DCM for 10 minutes, and a solution of t-BuOOH (150 μL 5.0-6.0 M solution in decane) was added to the mixture and agitated for 1 hour. The resin was washed with DCM (2 x 5 mL) and DMF (2 x 5 mL). Analytical reversed phase HPLC (t_R = 23.7 min). MS (ESI) *m/z* calculated for $C_{73}H_{92}N_{15}O_{19}P$: 1513.6 $(M+H)^+$ and 757.8 $(M+2H)^{2+}$. Found *m/z*: 758.1 $(M+2H)^{2+}$.

Ac-EEEHV-phospho-(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosyl SFPNKQK-CONH$_2$. The peptide sequence was completed by standard Fmoc SPPS in the dark. Then *N*-terminus then was acetyl capped with acetic anhydride (5 mL, 20% solution in DMF) and DIEA (3 mL, 0.195 M in DMF) for 20 min. Analytical reversed phase HPLC (t_R = 19.8 min, 95 %). MS (ESI) *m/z* calculated for $C_{83}H_{118}N_{21}O_{29}P$: 1903.81 $(M+H)^+$, 952.9 $(M+2H)^{2+}$, and 635.6 $(M+3H)^{3+}$. Found *m/z*: 953.4 $(M+2H)^{2+}$ and 636.0 $(M+3H)^{3+}$.

Magic Angle Spinning NMR. For the acquisition of magic angle spinning NMR spectra, aminomethylated polystyrene resin (0.85 mmole/gram loading; 1% DVB) was used for its higher loading. Again, standard Fmoc-SPPS protocol was followed for the synthesis of peptide trimer Ser-Pro-Gly, and the modifications made on bead were as described above for serine phosphitylation. ^{31}P MAS NMR (202.3 Hz, 2500 Hz, $CDCl_3$): free serine: no signal; phosphite: δ: 140.2ppm (d, J= 86.4 Hz); phosphotriester: δ: (-)1.7ppm (s); phosphodiester: δ: 0.4ppm (s).

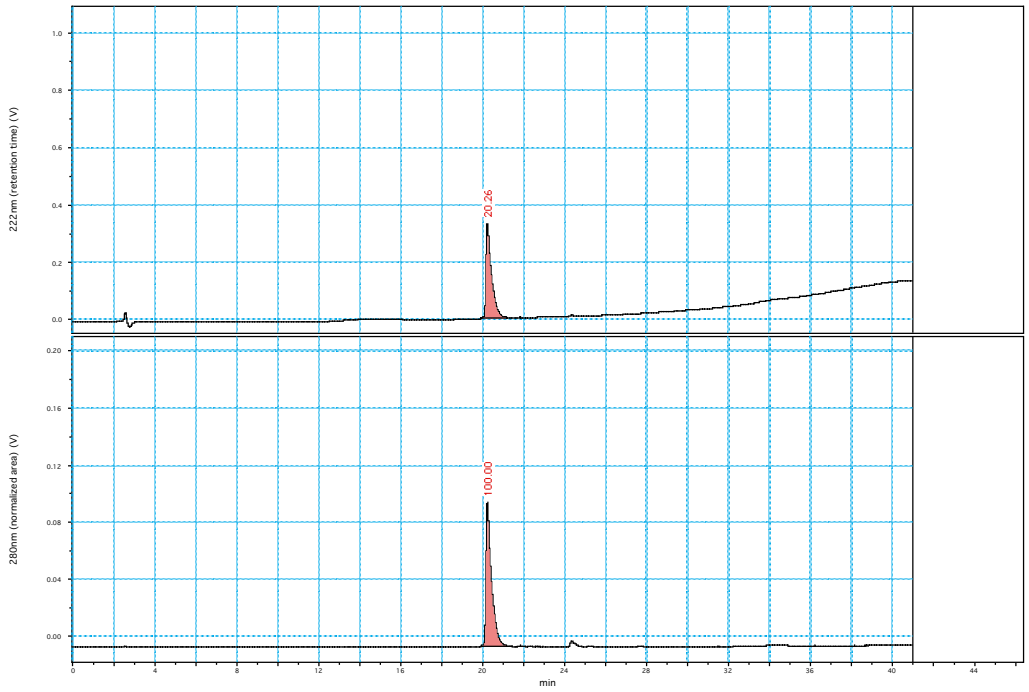
HPLC Traces of Purified Caged Peptides:

cpErk: Gradient: 93:7 to 0:100 (water:acetonitirile with 0.1% trifluoroacetic acid (TFA)) over 40 minutes at 1.0 mL per minute (t_R = 24.58 min).

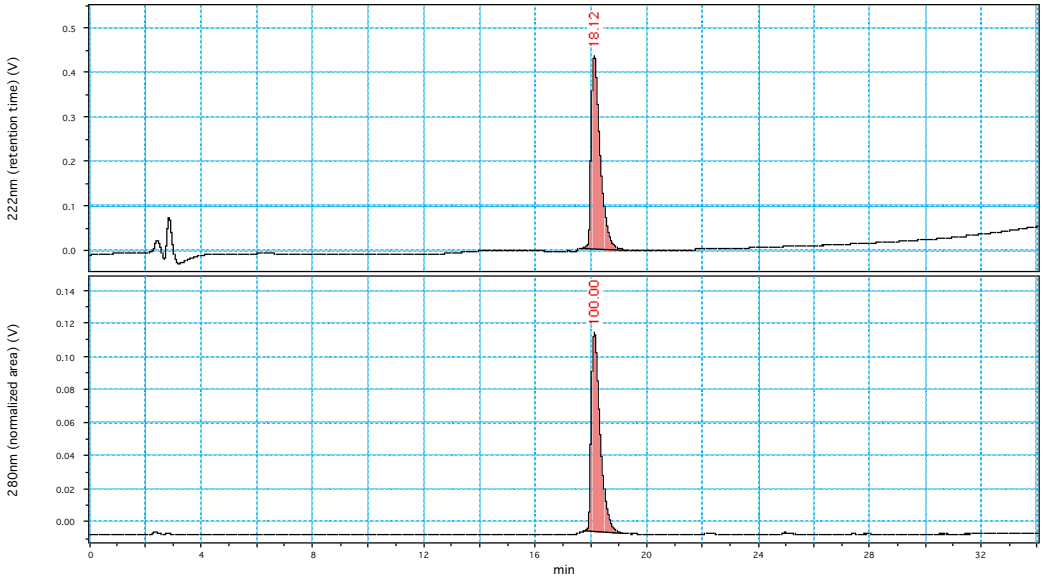


cpChk2:

Gradient: 93:7 to 0:100 (water:acetonitirile with 0.1% trifluoroacetic acid (TFA)) over 40 minutes at 1.0 mL per minute (t_R = 20.26).



cpPax:
Gradient: 93:7 to 0:100 (water:acetonitirile with 0.1% trifluoroacetic acid (TFA)) over 40 minutes at 1.0 mL per minute (t_R = 18.12 min).



Data for Quantum Yield Calculations:

Samples irradiated with a UVP UV Transilluminator, 365 nm, 7330 μW/cm^2 at the surface, in vessels with 1mm pathlength.

The quantum yield of each peptide was determined by comparison to the known quantum yield of caged phosphate as previously reported (Ellis-Davies, G. C. R.; Kaplan, J. H. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 187-191). An A_{350} value was measured for each sample at a 1mm path length in water, pH 7.1. Below is a sample calculation for cpERK.

$$\Phi_{cpERK} = \Phi_{cp} \times \frac{\%\Delta cpERK}{\%\Delta cp} \times \frac{A_{350}\,cp}{A_{350}cpERK}$$

$$\Phi_{cpERK} = 0.54 \times \frac{15.134\%}{17.3\,\%} \times \frac{0.01911}{0.03506}$$

$$\Phi_{cpERK} = 0.2568$$

$$\Phi_{cpERK} = 0.26$$

cpERK: Analytical HPLC gradient used for caged phosphate and caged peptide: 95:5 to 30:70 (water:acetonitrile with 0.1% TFA) over 40 minutes at 1.0 mL per minute.

Data Tables for **cpERK**

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	3.30	0.641	1.000	----
cpERK (t=0)	30.47	24.664	38.477	0.03506
Inosine (t=15s)	3.37	0.713	1.000	----
cpERK (t=15s)	30.58	22.939	32.173	----

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	3.36	0.559	1.000	-----
Caged P (t=0)	14.91	16.737	29.941	0.01911
Inosine (t=15s)	3.21	0.686	1.000	-----
Caged P (t=15s)	14.69	16.739	24.401	-----

cpChk2: Analytical HPLC gradient used for caged phosphate and caged peptide: 93:7to 65:35 (water:acetonitrile with 0.1% TFA) over 15 minutes, then 65:35 to 40:60 over 20 minutes at 1.0 mL per minute.

Data Tables for **cpChk2**

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	2.97	1.45	1.000	----
cpChk2 (t=0)	21.33	3.34	2.2942	0.02516
Inosine (t=15s)	2.93	1.50	1.000	----
cpChk2 (t=15s)	21.28	2.95	1.9609	----

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	2.95	1.44	1.000	-----
Caged P (t=0)	18.37	1.91	1.3341	0.02119
Inosine (t=15s)	2.86	1.43	1.000	-----
Caged P(t=15s)	18.35	1.72	1.0690	-----

cpPax: Analytical HPLC gradient used for caged phosphate and caged peptide: 93:7 to 25:75 (water:acetonitrile with 0.1% TFA) over 30 at 1.0 mL per minute.

Data Tables for **cpPax**

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	3.06	0.433	1.000	----
cpPax (t=0)	20.19	0.566	1.306	0.01588
Inosine (t=15s)	3.06	0.467	1.000	----
cpPax (t=15s)	30.58	0.532	1.139	----

Peak Name	Retention Time (minutes)	Average Area	Average Normalized Area	Average A_{350} in water, pH 7.1
Inosine (t=0)	3.36	0.523	1.000	-----
Caged P (t=0)	20.09	0.476	0.910	0.01825
Inosine (t=15s)	3.7	0.607	1.000	-----
Caged P (t=15s)	20.03	0.393	0.647	-----