# Supporting Information

**Unambiguous Identification of Serine and Threonine Pyrophosphorylation Using Neutral-Loss-Triggered Electron-Transfer/Higher-Energy Collision Dissociation (EThcD)**

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# Supplementary Figures

**Representative HCD MS/MS spectra**

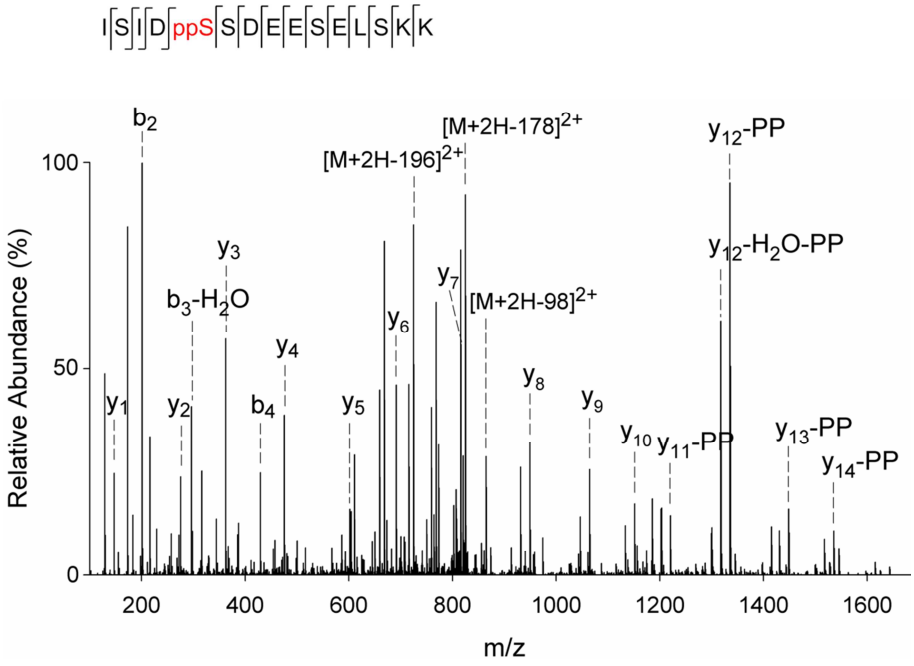



Figure S1. HCD MS/MS spectrum of doubly charged peptide ISIDppSSDEESELSKK (**PP-4**)

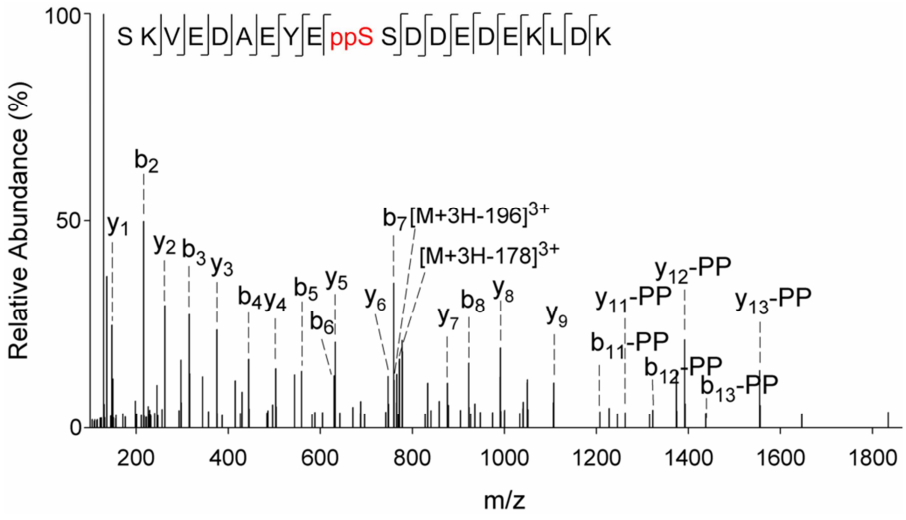



Figure S2. HCD MS/MS spectrum of triply charged peptide SKVEDAEYEppSSDDEDEKLDK (**PP-6**)

**Representative CID MS/MS spectra**

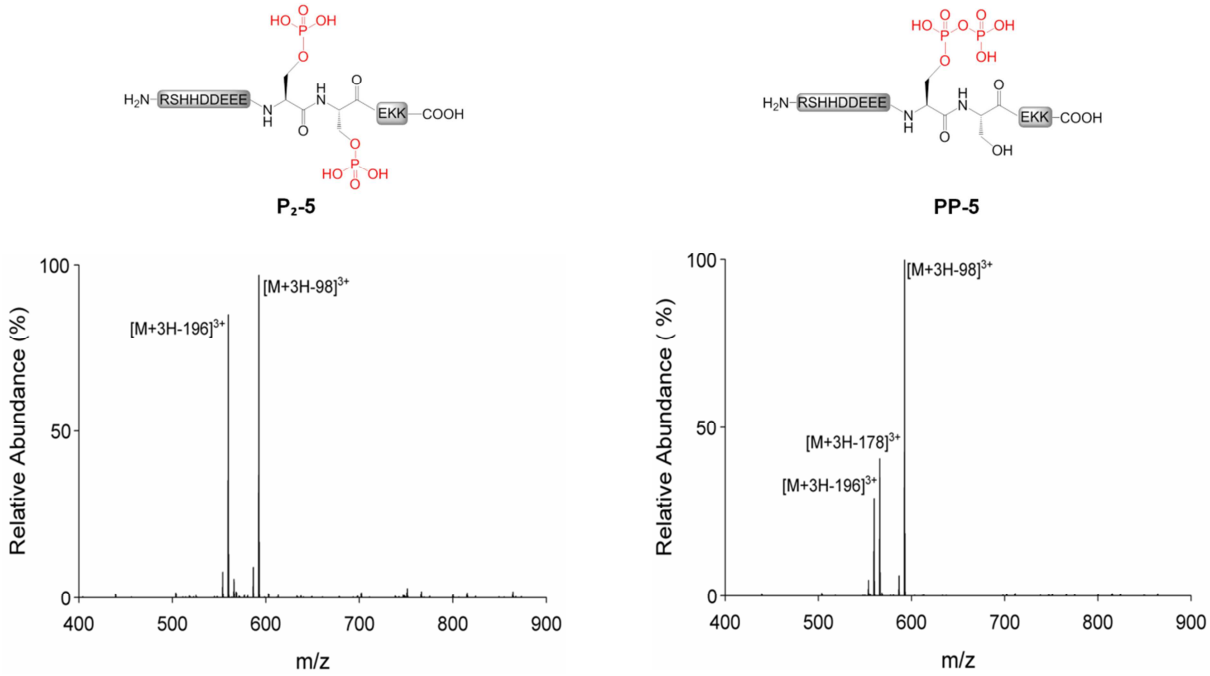



Figure S3. Comparison of CID MS/MS spectrum of triply charged peptide RSHHDDEEEpSpSEKK (**$P_2$-5**) and peptide RSHHDDEEEppSSEKK (**PP-5**)

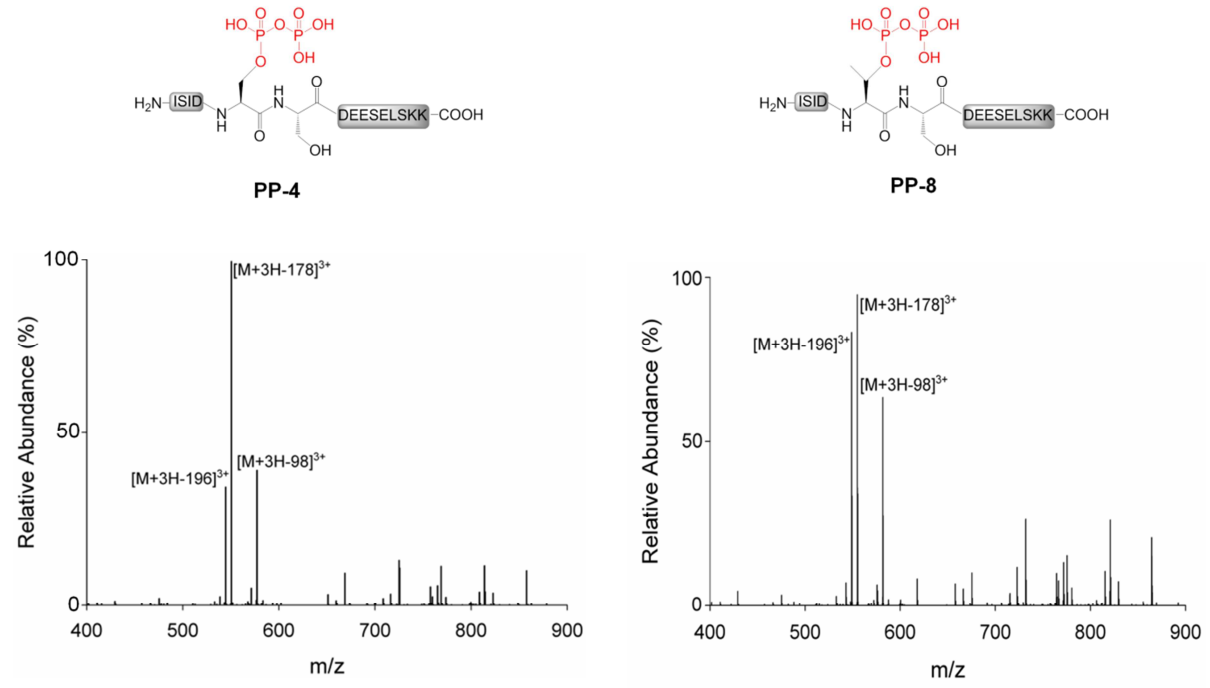



Figure S4. Comparison of CID MS/MS spectrum of triply charged peptide ISIDppSSDEESELSKK (**PP-4**) and peptide ISIDppTSDEESELSKK (**PP-8**)

**Representative EThcD MS/MS spectra of triply charged precursor ions**

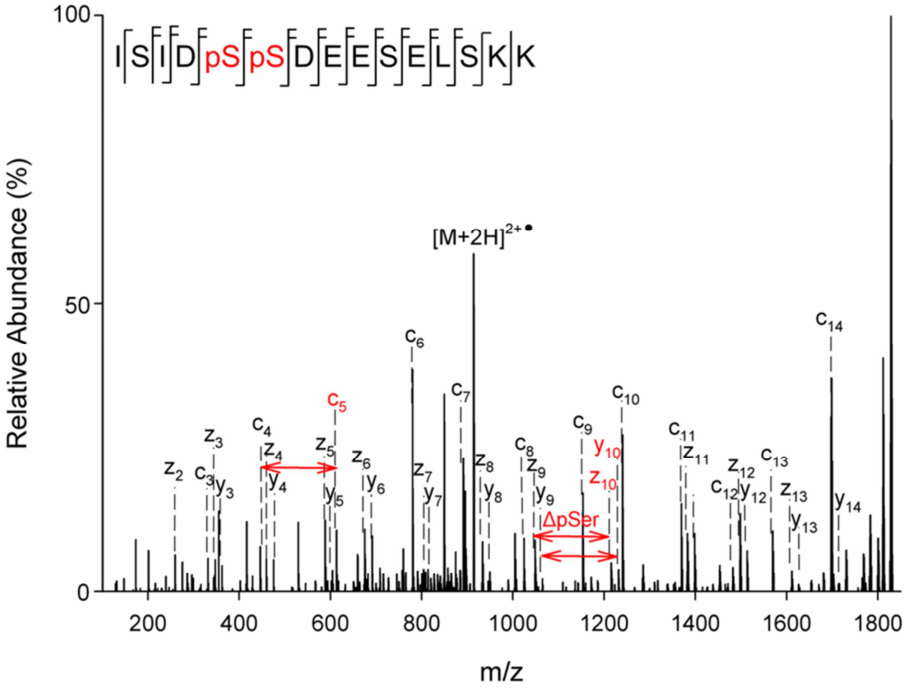



Figure S5. EThcD MS/MS spectrum of triply charged peptide ISIDpSpSDEESELSKK (**$P_2$-4**)

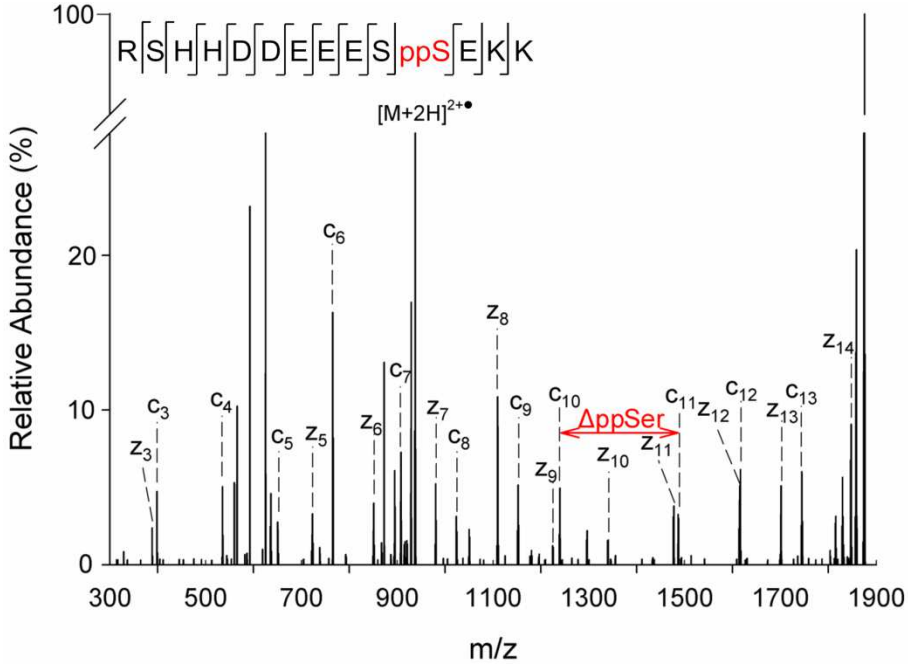



Figure S6. EThcD MS/MS spectrum of triply charged peptide RSHHDDEEESppSEKK (**PP-5**)

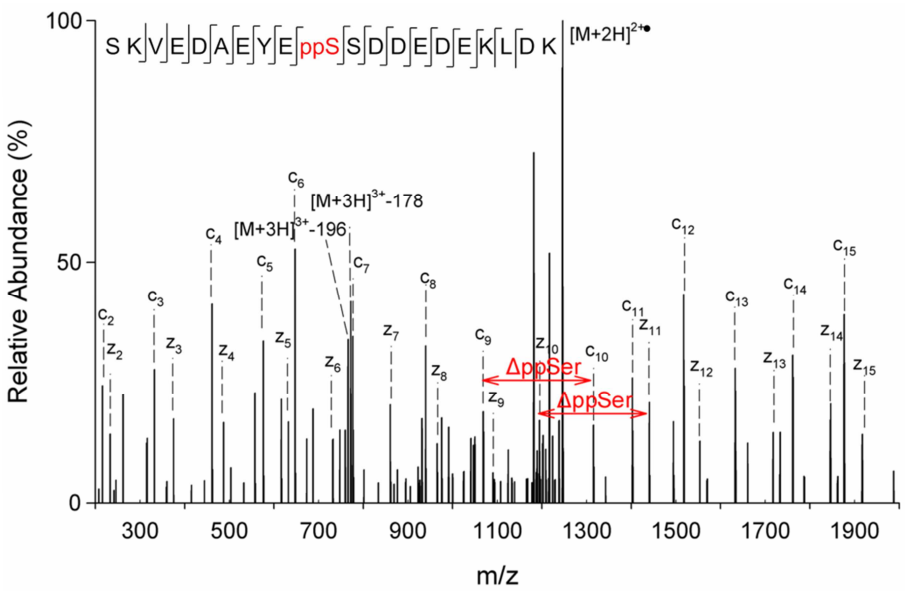



Figure S7. EThcD MS/MS spectrum of triply charged peptide SKVEDAEYEppSSDDEDEKLDK (**PP-6**)

**Representative EThcD MS/MS spectra of doubly charged precursor ions**

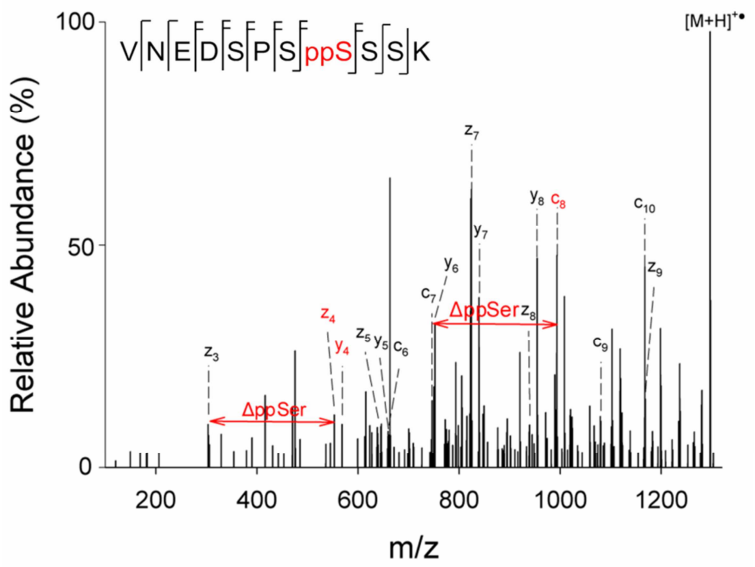



Figure S8. EThcD MS/MS spectrum of doubly charged peptide VNEDSPSppSSSK (**PP-1**)

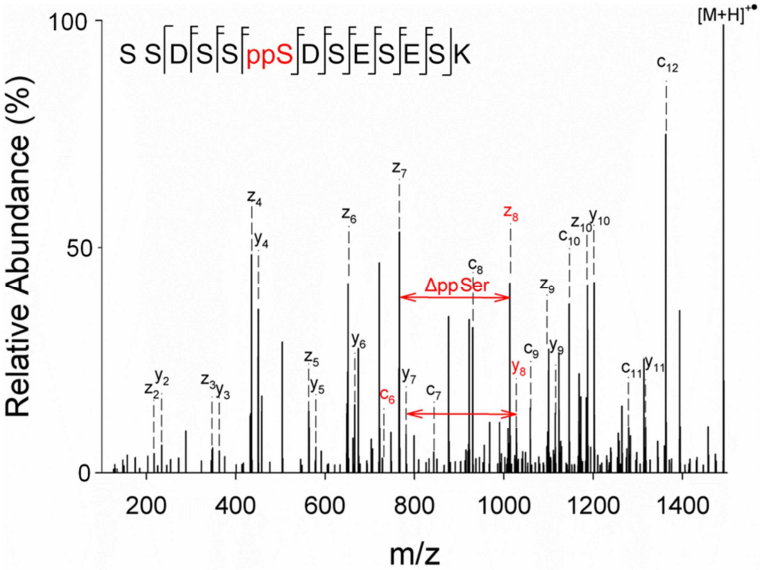



Figure S9. EThcD MS/MS spectrum of doubly charged peptide SSDSSppSDSESESK (**PP-3**)

**Chromatographic behavior of di-and pyrophosphorylated peptides**

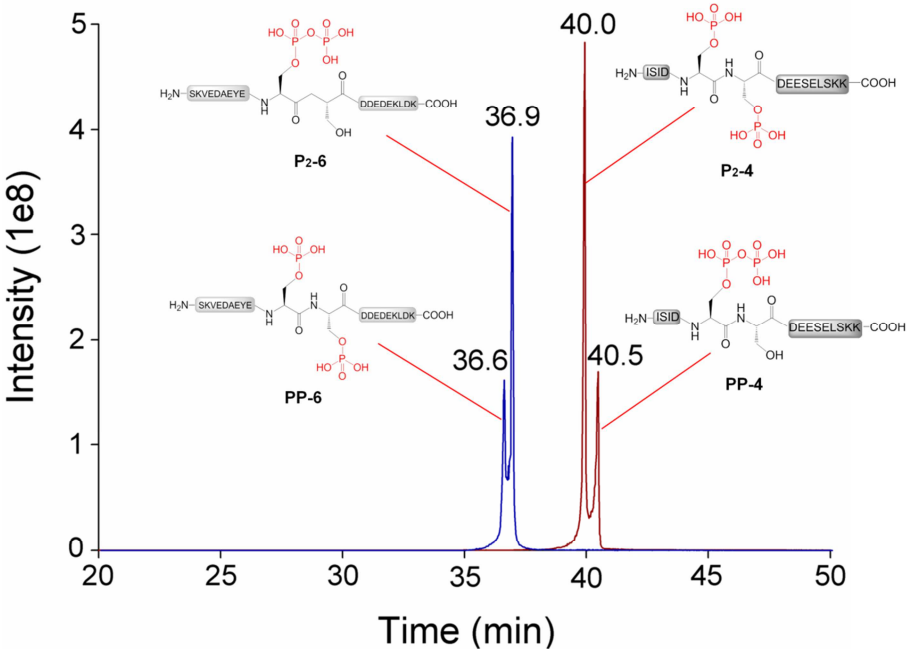



Figure S10. Extracted ion chromatograms (XIC) of the synthetic peptides **$P_2$-6**, **PP-6** (m/z 830.982; blue line), **$P_2$-4** and **PP-4** (*m/z* 913.873; red line) showing the similar chromatographic behavior of di- and pyrophosphorylated peptides. The doubly phosphorylated peptides exhibit shorter retention times than the pyrophosphorylated counterpart.

**Spike-in experiment peptide ISIDppTSDEESELSKK (PP-8)**

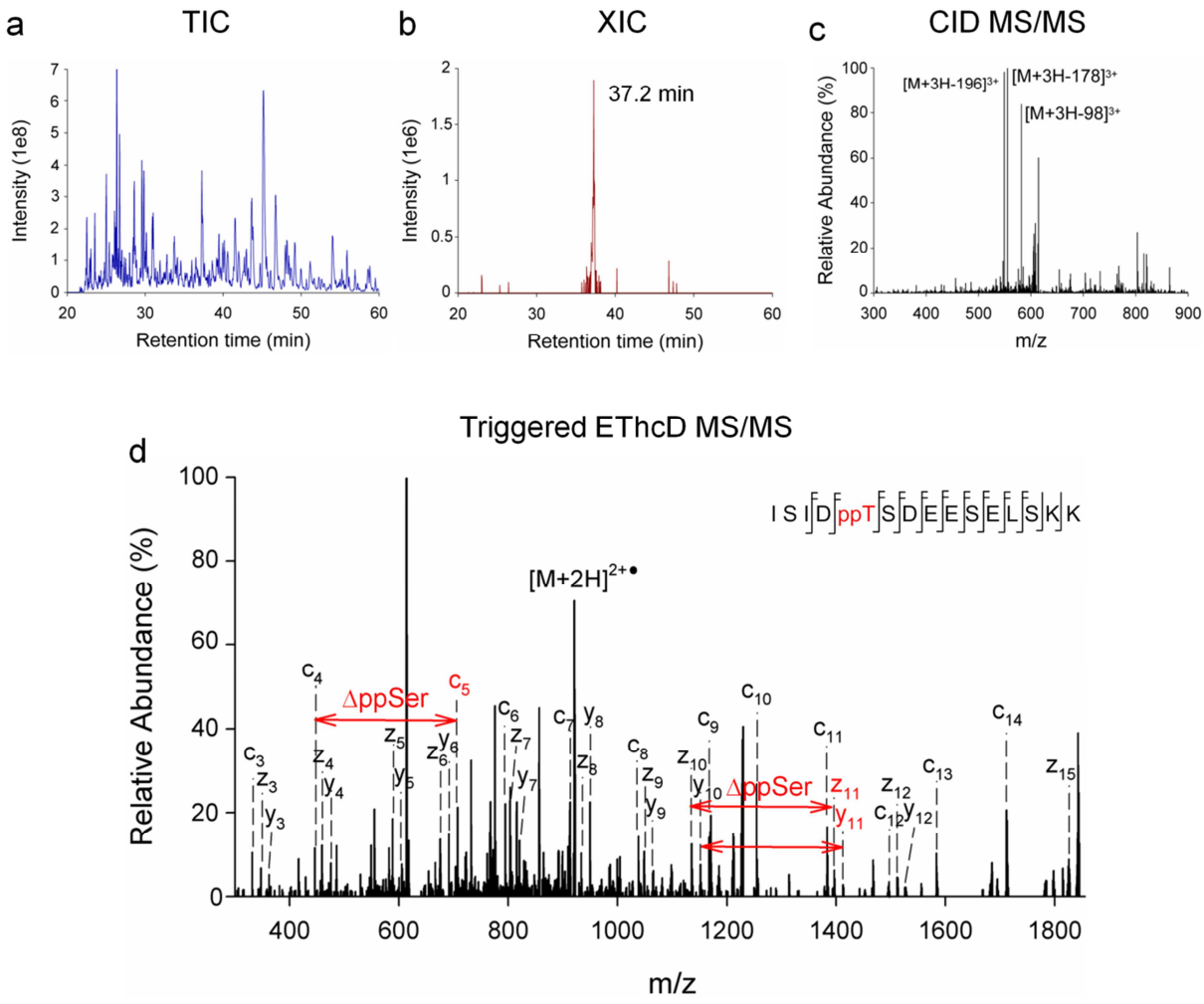
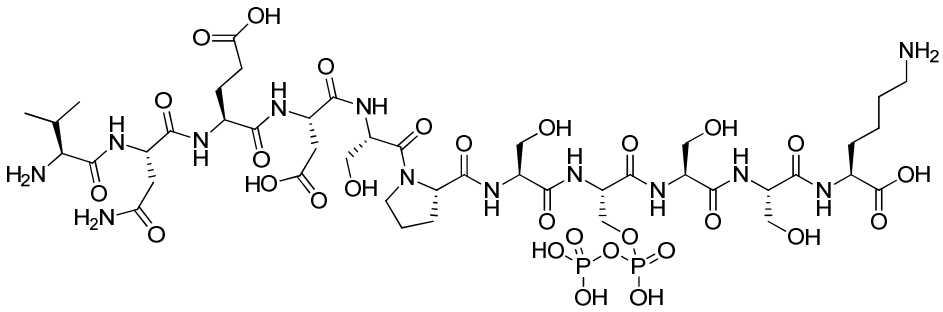


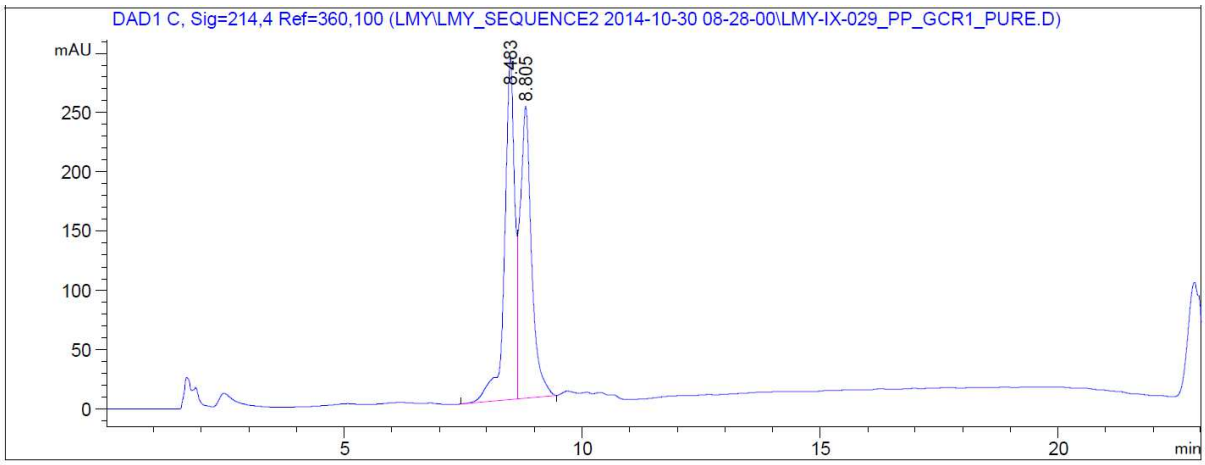

Figure S11. Detection of a synthetic pyrophosphopeptide in a spike-in experiment using the DDNL-EThcD approach. (a) Total ion chromatogram (TIC) of the HeLa protein digest. (b) Extracted ion chromatogram (XIC) *m/z* 614.255 of the synthetic peptide **PP-8**. (c) CID MS/MS spectrum of peptide **PP-8** acquired at a retention time of 37.23 min indicating dominant neutral losses of 98, 178 and 196 Da. (d) Triggered EThcD MS/MS spectrum of the pyrophosphorylated peptide ISIDppTSDEESELSKK showing gapless sequence coverage without loss of the labile modification. Fragment ions pinpointing the site of modification are labled in red.

**Characterization of synthetic pyrophophopeptides**

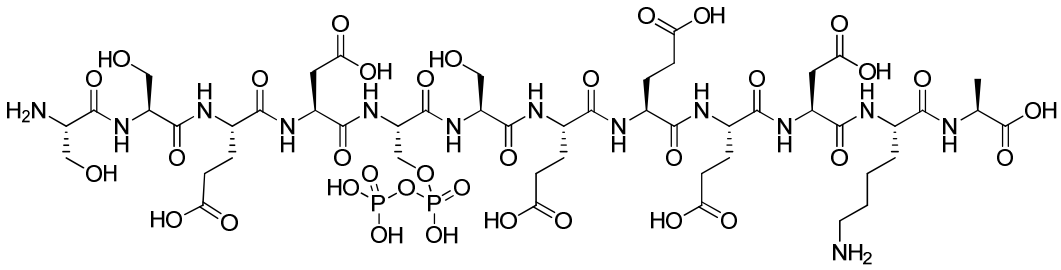
Peptide **PP-1** (VNEDSPSppSSSK)



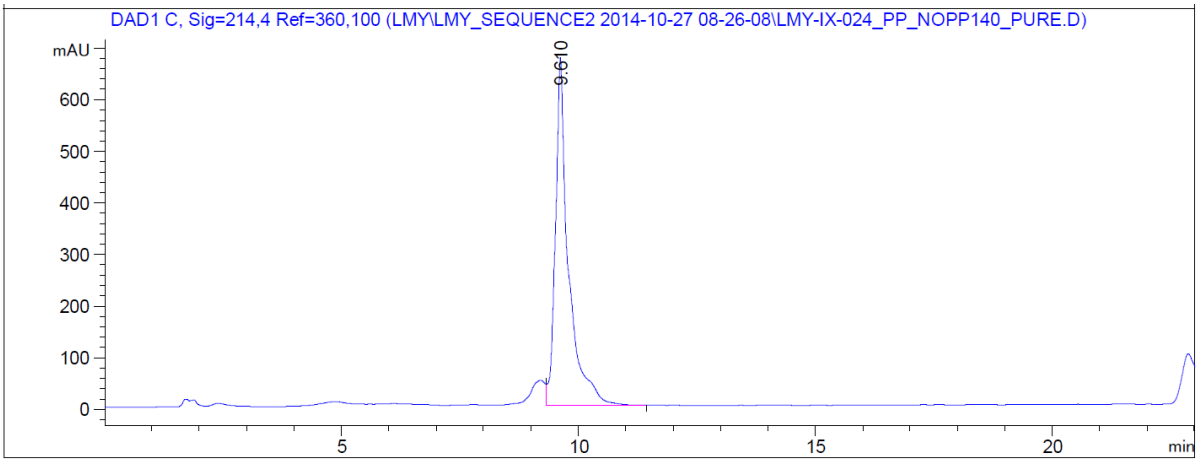
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A, t = 20 min 20% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 8.483 min & $T_R$ = 8.805 min]. Both peaks in the analytical HPLC were collected and HRMS confirmed that both fractions contained pyrophosphopeptide **PP-1** as the primary product (HRMS (Bulk Material) $[M+H]^+$ calcd for $C_{44}H_{76}N_{13}O_{28}P_2^+$ 1296.4393, found 1296.4463; $[M+2H]^{2+}$ calcd for $C_{44}H_{77}N_{13}O_{28}P_2^{2+}$ 648.7233, found 648.7255; $[M+2H+Na]^{3+}$ calcd for $C_{44}H_{77}N_{13}NaO_{28}P_2^{3+}$ 440.8133, found 440.8070) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 7.70) δ -6.78 (d, $^2J_{P\text{-}P}$ = 27.8 Hz), -10.73 (d, $^2J_{P\text{-}P}$ = 19.9 Hz).



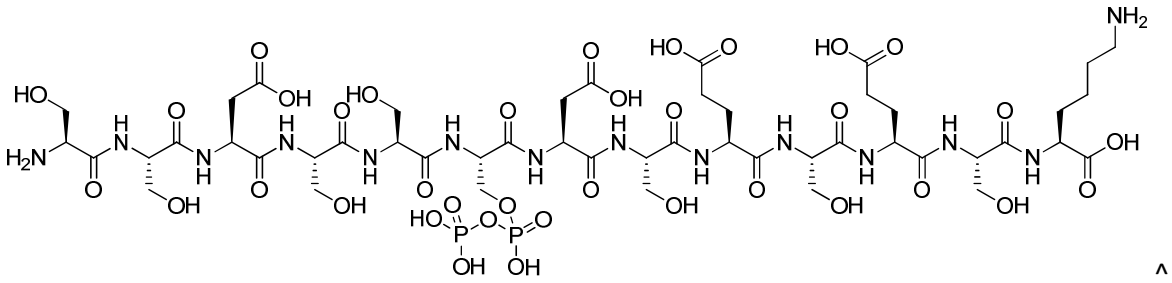
Peptide **PP-2** (SSEDppSSEEEDKA)



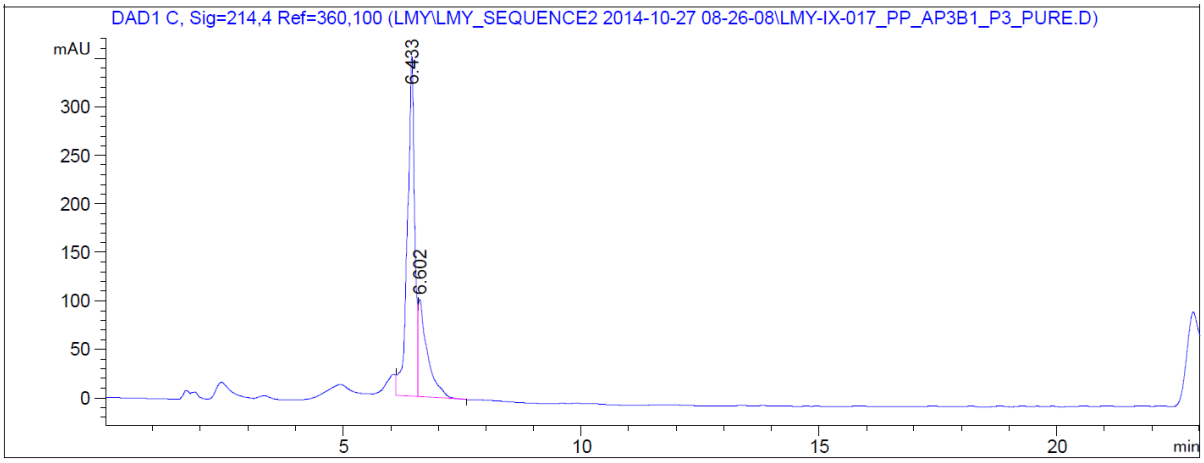
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A, t = 20 min 20% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 9.610 min], and the identity of pyrophosphopeptide **PP-2** was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{49}H_{80}N_{13}O_{35}P_2^+$ 1472.4350, found 1472.4332; $[M+2H]^{2+}$ calcd for $C_{49}H_{81}N_{13}O_{35}P_2^{2+}$ 736.7211, found 736.72125; $[M+2H+Na]^{3+}$ calcd for $C_{49}H_{81}N_{13}NaO_{35}P_2^{3+}$ 499.4794, found 499.4697) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 7.59) δ -6.84 (d, $^2J_{P\text{-}P}$ = 20.5 Hz), -10.87 (d, $^2J_{P\text{-}P}$ = 21.1 Hz).



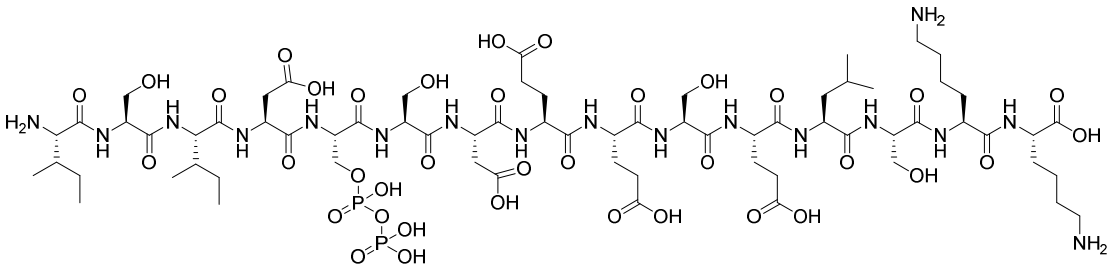
Peptide **PP-3** (SSEDppSSEEEDKA)



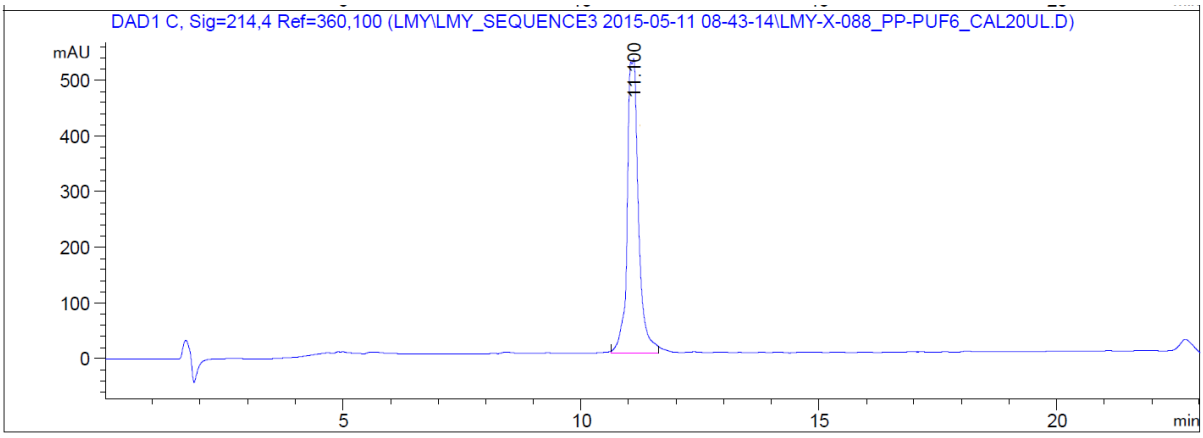
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A, t = 20 min 20% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 6.433 min], and the identity of the pyrophosphopeptide **PP-3** was confirmed by mass spectrometry (HRMS $[M+H+Na]^{2+}$ calcd for $C_{48}H_{81}N_{14}O_{36}P_2^+$ 1491.4408, found 1491.4410; $[M+2H]^{2+}$ calcd for $C_{48}H_{82}N_{14}O_{36}P_2^{2+}$ 746.2240, found 746.2255; $[M+2H+Na]^{3+}$ calcd for $C_{48}H_{82}N_{14}NaO_{36}P_2^{3+}$ 505.8147, found 505.8057) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 7.67) δ -6.57 (d, $^2J_{P\text{-}P}$ 21.8 Hz), -10.81 (d, $^2J_{P\text{-}P}$ = 21.1 Hz).



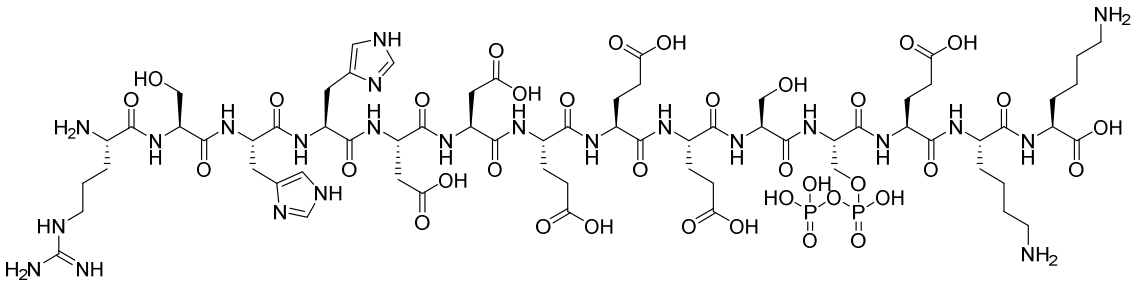
Peptide **PP-4** (ISIDppSSDEESELKK)



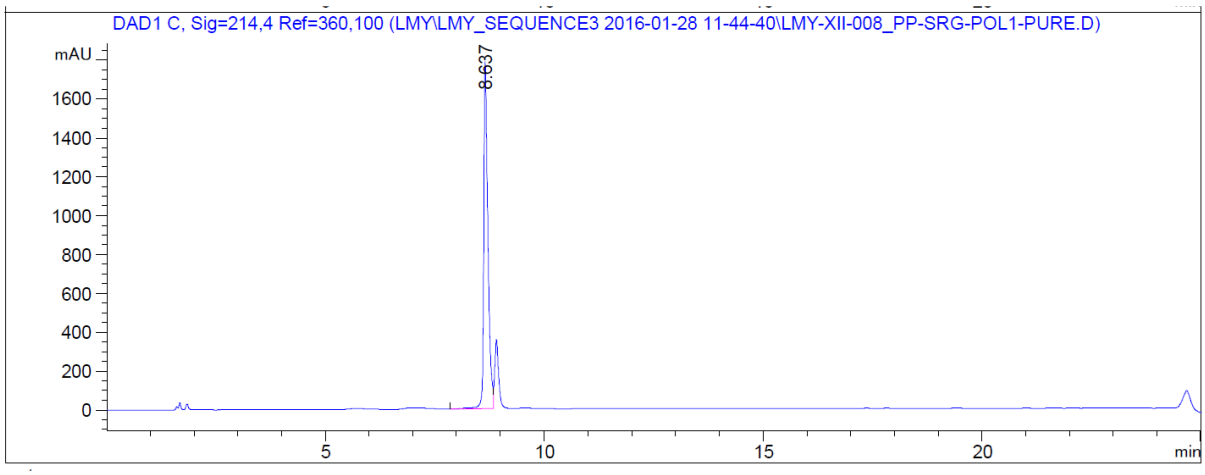
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 47% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 13.957 min], and the identity of pyrophosphopeptide **PP-4** was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{68}H_{119}N_{17}O_{37}P_2^{2+}$ 913.8709, found 913.8686; $[M+3H]^{3+}$ calcd for $C_{68}H_{119}N_{17}O_{37}P_2^{2+}$ 609.5830, found 609.5823) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 8.07) δ -6.77 (d, $^2J_{P\text{-}P}$ = 20.5 Hz), -10.99 (d, $^2J_{P\text{-}P}$ = 21.7 Hz).



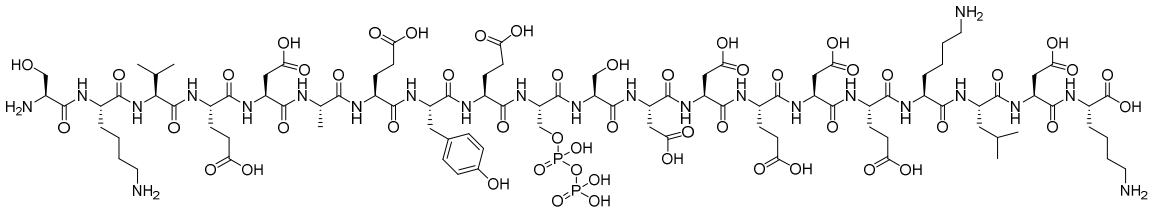
Peptide **PP-5** (RSHHDDEEESppSEKK)



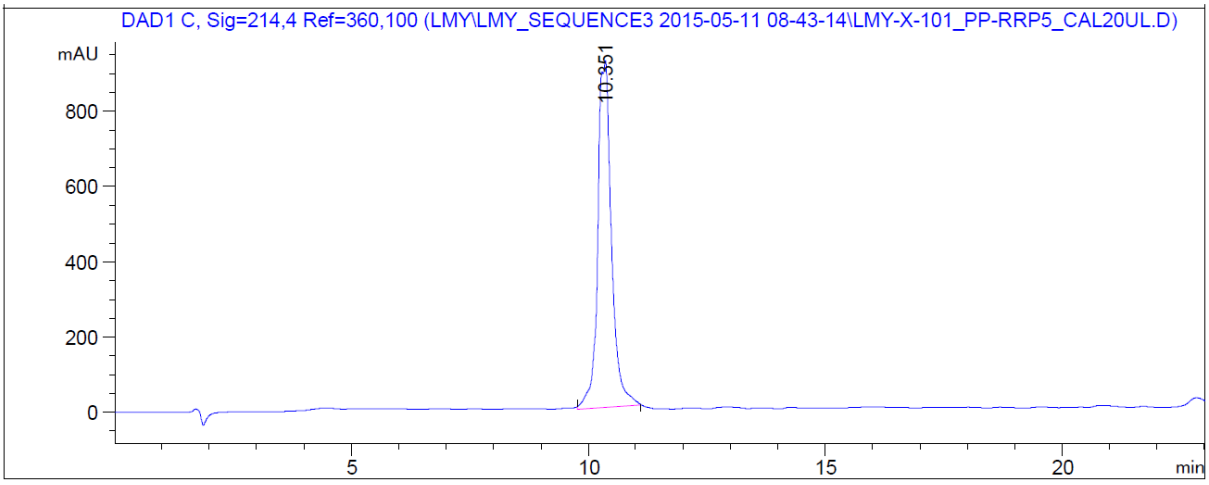
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 8.637 min], and the identity of pyrophosphopeptide **PP-5** was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{95}H_{151}N_{23}O_{51}P_2^{2+}$ 936.8362, found 936.8294; $[M+3H]^{3+}$ calcd for $C_{95}H_{152}N_{23}O_{51}P_2^{3+}$ 624.8908, found 624.8833; $[M+Na+3H]^{4+}$ calcd for $C_{95}H_{152}N_{23}NaO_{51}P_2^{4+}$ 468.9181, found 468.9133) and $^{31}P$ NMR (202 MHz, $H_2O$, pH = 7.91) δ -6.27 (d, , $^2J_{P\text{-}P}$ = 21.8 Hz), -10.76 (d, , $^2J_{P\text{-}P}$ = 21.4 Hz).



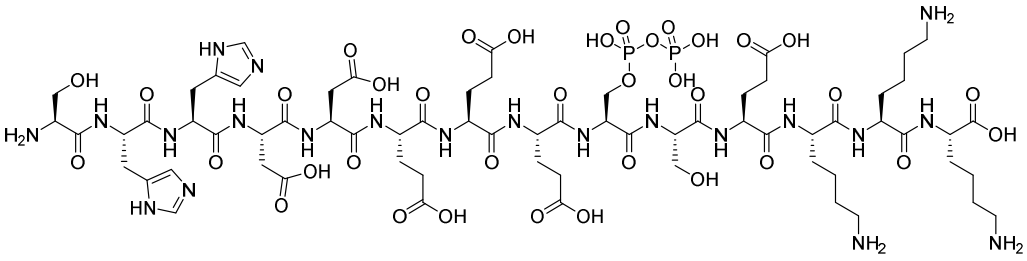
Peptide **PP-6** (SKVEDAEYEppSSDDEDEKLDK)



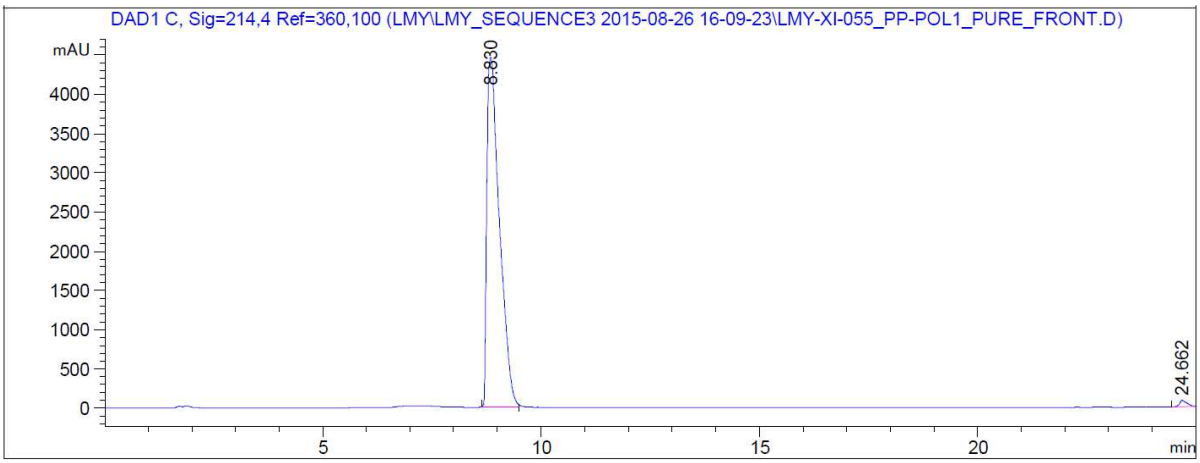
The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 47% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 10.351 min], and the identity of pyrophosphopeptide **PP-6** was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{95}H_{151}N_{23}O_{51}P_2^{2+}$ 1246.4714, found 1246.4687; $[M+3H]^{3+}$ calcd for $C_{95}H_{152}N_{23}O_{51}P_2^{3+}$ 831.3167, found 831.3180; $[M+Na+3H]^{4+}$ calcd for $C_{95}H_{152}N_{23}NaO_{51}P_2^{4+}$ 629.2348, found 629.7307) and $^{31}P$ NMR (202 MHz, $H_2O$, pH = 7.94) δ -6.64 (d, , $^2J_{P\text{-}P}$ = 21.7 Hz), -10.78 (d, , $^2J_{P\text{-}P}$ = 21.5 Hz).



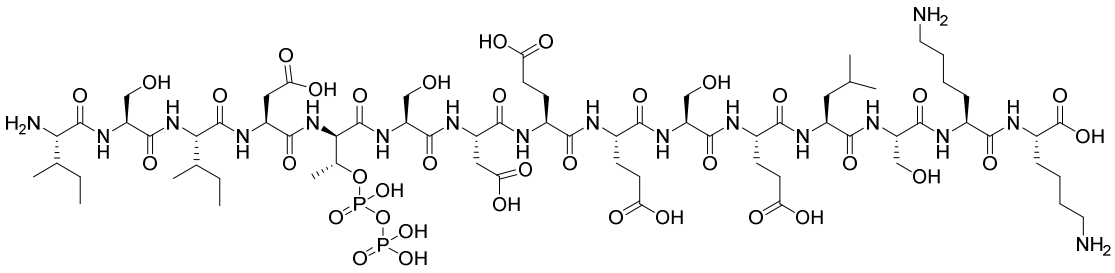
Peptide **PP-7** (SHHDDEEESppSEKKK)



Using Procedure B, pyrophosphopeptide **xxx** was prepared from phosphopeptide **xxx** (11.5 mg, 6.52 μmol) in 2 steps as previously described[5] to give the title peptide in 28% yield (3.40 mg, 1.84 μmol) over two steps as a white solid. The purity of the isolated peptide was determined by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 30% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 8.830 min], and the identity of pyrophosphopeptide **PP-7** was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{67}H_{109}N_{21}O_{36}P_2^{2+}$ 922.8404, found 922.8409; $[M+3H]^{3+}$ calcd for $C_{67}H_{110}N_{21}O_{36}P_2^{3+}$ 615.5627, found 615.5619; $[M+4H]^{4+}$ calcd for $C_{67}H_{111}N_{21}O_{36}P_2^{4+}$ 461.9238, found 461.9221) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 7.75) δ -6.43 (d, $^2J_{P\text{-}P}$ = 21.5 Hz), -10.86 (d, $^2J_{P\text{-}P}$ = 21.5 Hz).



Peptide **PP-8** (ISIDppTSDEESELSKK)



The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 47% of solvent B in solvent A; 1 mL/min; 214 nm; $T_R$ = 10.463 min], and the identity of pyrophosphopeptide **PP-8** was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{69}H_{121}N_{17}O_{37}P_2^{2+}$ 921.8820, found 921.0145; $[M+3H]^{3+}$ calcd for $C_{68}H_{119}N_{17}O_{37}P_2^{2+}$ 614.2549, found 614.3337) and $^{31}$P NMR (202 MHz, $H_2O$, pH = 7.91) δ -6.97 (d, $^2J_{P\text{-}P}$ = 20.4 Hz), -11.73 (d, $^2J_{P\text{-}P}$ = 20.5 Hz).