SUPPORTING INFORMATION

A Stable Pyrophosphoserine Analog for Incorporation into Peptides and Proteins

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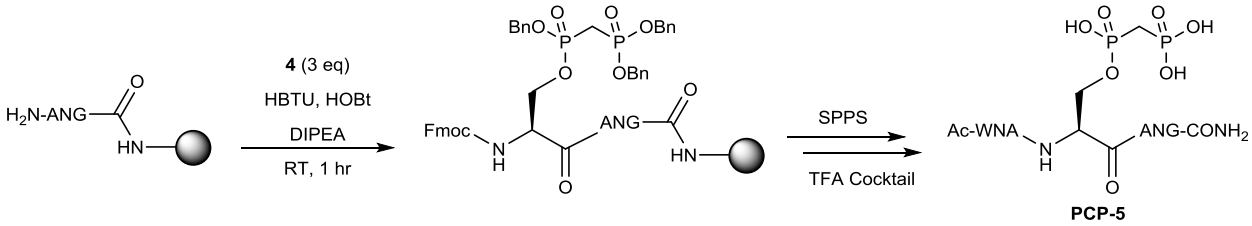


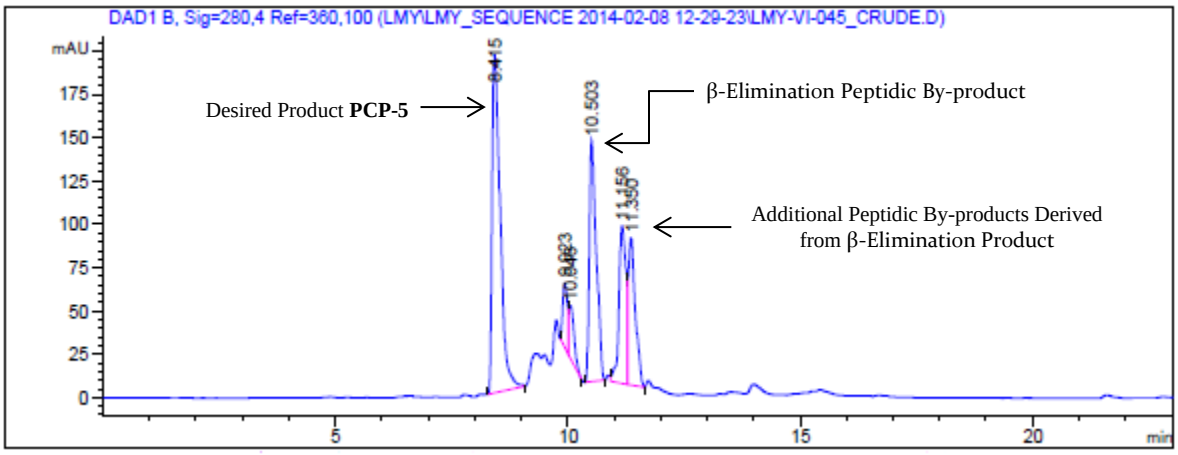
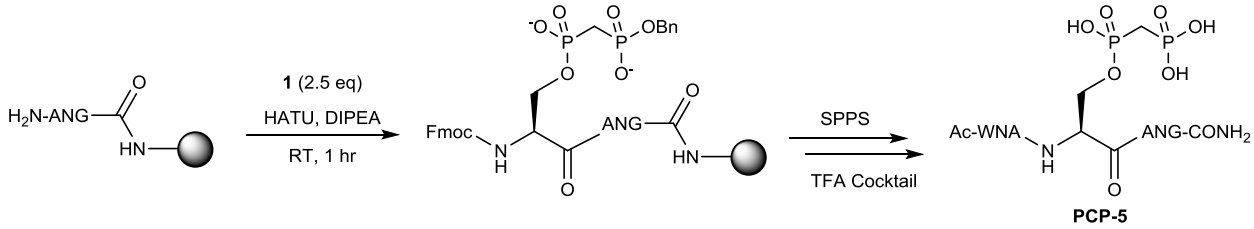


Figure S1. *Incorporation of Analog* ***4*** *by Solid-Phase Peptide Synthesis (SPPS).* Analytical HPLC analysis (280 nm absorbance) of crude product resulting from incorporation of monomer **4** to form peptide **PCP-5** indicated approximately 42% desired product **PCP-5** (T_R = 8.415 min, found $[M+H]^+$ = 918.2889) with significant by-product formation. The primary by-product (25% composition) at T_R = 10.503 min was identified by mass spectrometry (found $[M+H]^+$ = 742.3246) and determined to be the product resulting from β-elimination of the bisphosphonate moiety to form an alkene byproduct. Products with T_R = 11.156-11.350 min (33% composition) were isolated and checked by mass spectrometry (found $[M+H]^+$ = 827.4140 & m/z = 877.3673). These masses could not be assigned to any obvious by-product, but most likely formed as products resulting from addition into the alkene by-product. Abbreviations: HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt = hydroxybenzotriazole; DIPEA = *N*,*N*-diisopropylethylamine.



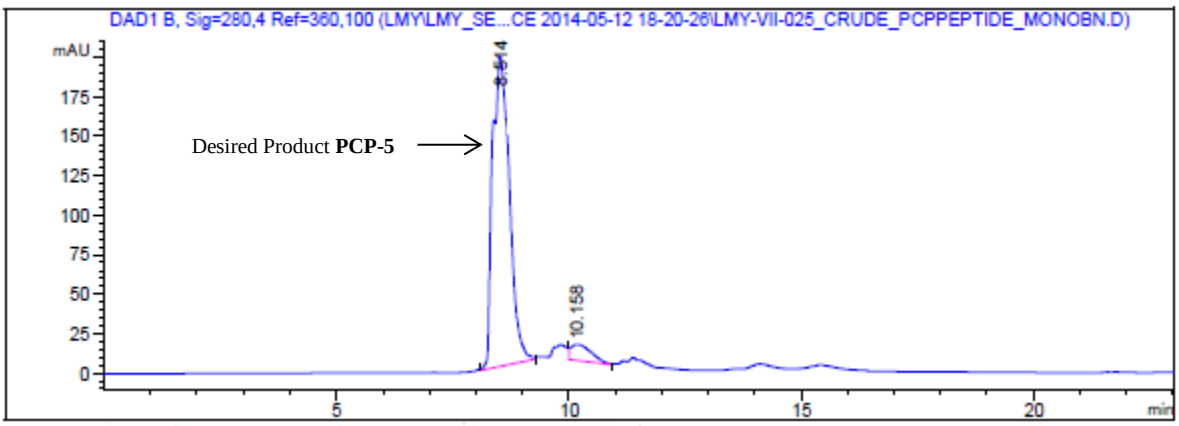


Figure S2. *Incorporation of Analog* ***1*** *by Solid-Phase Peptide Synthesis (SPPS).* Analytical HPLC analysis (280 nm absorbance) of crude product resulting from incorporation of monomer **1** indicated formation of product **PCP-5** (T_R = 8.514 min) with minimal to no by-product formation. The TFA Cocktail used was 95% trifluoroacetic acid, 2.5% H_2O, and 2.5% triisopropylsilane (RT, 4 hours). Abbreviations: HATU = 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine.

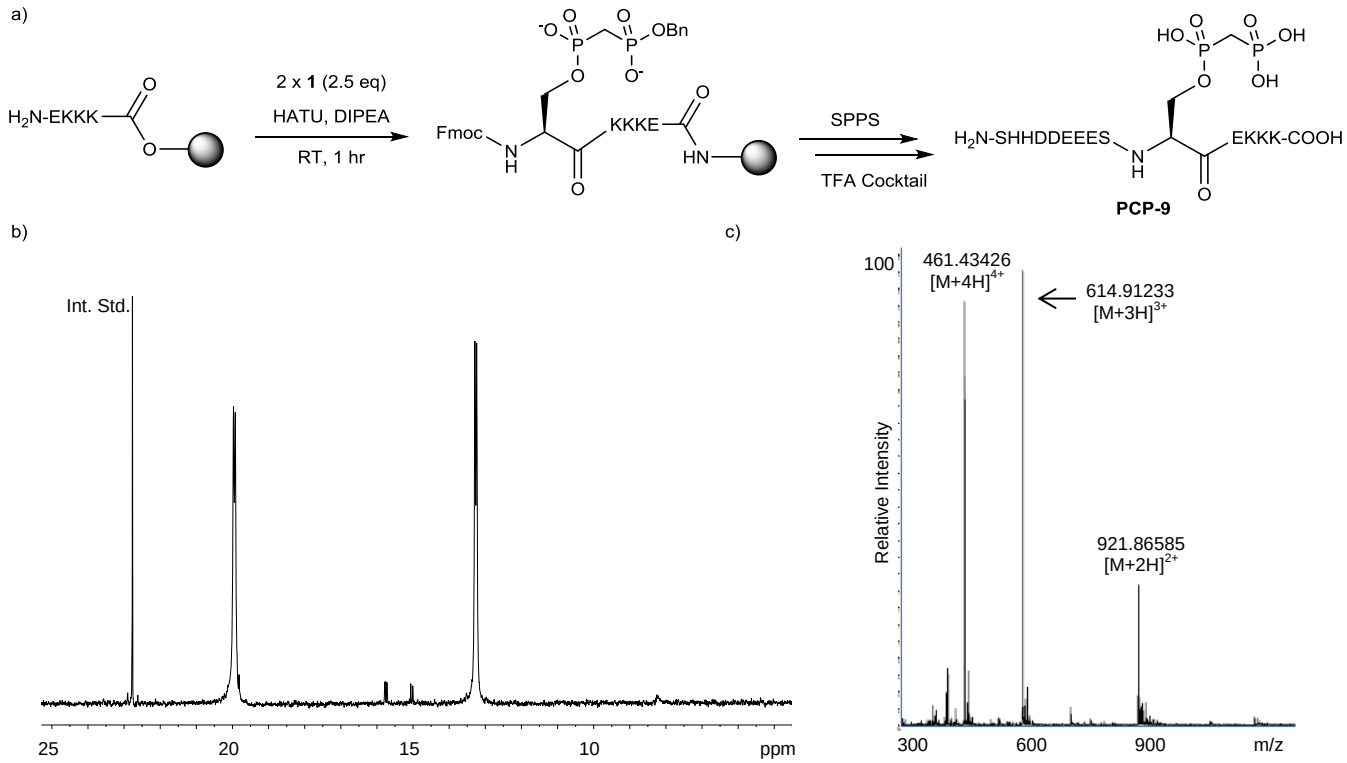


Figure S3. *Representative Characterization of Compound* ***PCP-9***. a) Solid-phase synthesis (SPPS) of peptide **PCP-9** incorporating monomer **1**. Note, for this substrate, the incorporation of stable analog **1** required a double-coupling using the standard optimized coupling conditions for full conversion to desired product **PCP-9** as indicated by the Kaiser test. See Chemical Synthesis Section for detailed preparation procedures for each peptide. b) ^{31}P NMR of HPLC purified peptide **PCP-9** at pH = 8.10, referenced to internal standard tetramethylphosphonium bromide (22.77 ppm). c) HRMS $[M+2H]^{2+}$, $[M+3H]^{3+}$, and $[M+4H]^{4+}$ of HPLC purified peptide **PCP-10**. See Peptide HPLC Traces & Mass Spectrometry Characterization and NMR Spectra Sections for characterization of all peptides. The TFA Cocktail used was 95% trifluoroacetic acid, 2.5% H_2O, and 2.5% triisopropylsilane (RT, 4 hours). Abbreviations: HATU = 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine.

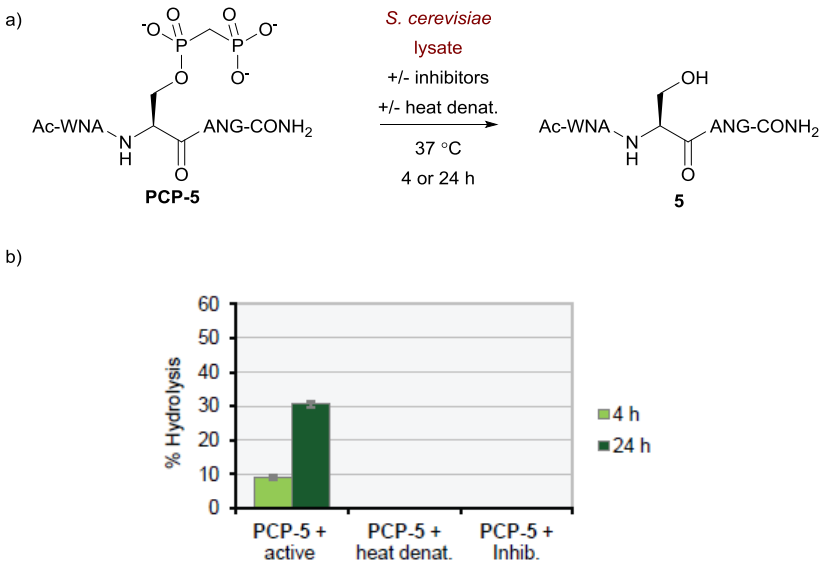


Figure S4. a) General reaction scheme for lysate stability studies of **PCP-5** in the presence of active lysate, heat denatured lysate, and lysate with common phosphatase inhibitors. Phosphatase inhibitors include 2 mM Ethylenediaminetetraacetic acid (EDTA) and 5% phosphatase cocktail 2 (Sigma Aldrich). b) No hydrolysis of **PCP-5** in the heat denatured lysate or in the presence of common phosphatase inhibitors is observed after 4 and 24 hour of incubation at 37 ˚C while hydrolysis is observed in the active lysate indicating that the methylene-bisphosphonate moiety is enzymatically removed. Error bars represent the standard deviation for triplicate experiments.

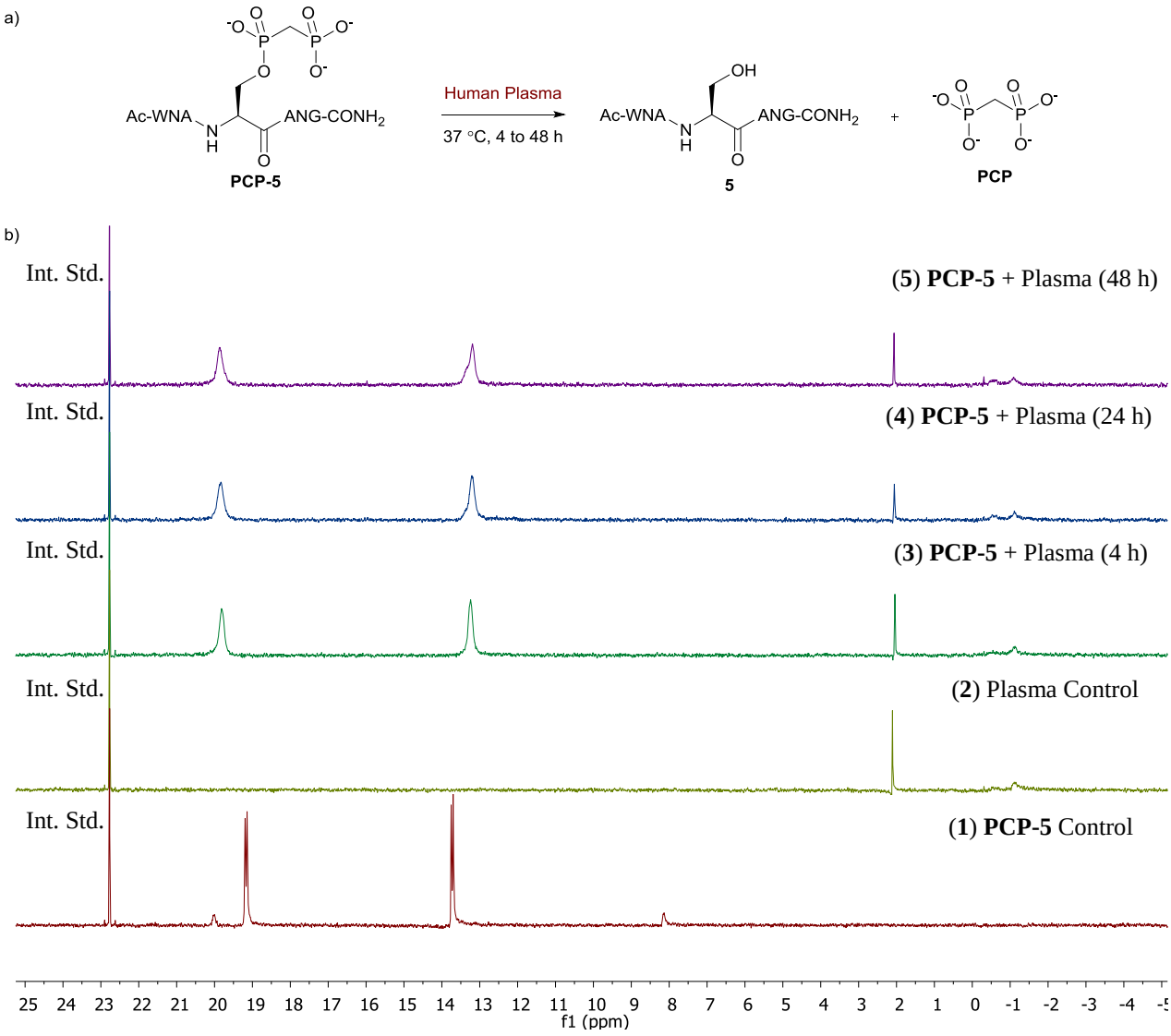


Figure S5. a) General reaction scheme for mammalian plasma stability study of **PCP-5**. b) ^{31}P NMR traces of (1) **PCP-5** in Tris-HCl buffer (pH 7.50) control, (2) human plasma (Sigma Aldrich) in Tris-HCl buffer control, (3) **PCP-5** in buffered plasma after 4 hours incubation at 37 ˚C, (4) **PCP-5** in buffered plasma after 24 hours incubation at 37 ˚C, and (5) **PCP-5** in buffered plasma after 48 hours incubation at 37 ˚C. The methylene-bisphosphonate moiety is resistant to hydrolysis in plasma after 48 hours of incubation. If the bisphosphonate moiety were hydrolyzing, the **PCP** byproduct would be visible as a singlet at 11.4 ppm,[1] which is not present in any of these traces. Note that the peaks between -2 and 2 ppm are monophosphorylated species which are already present in the plasma serum since they are also observed in the plasma control sample.

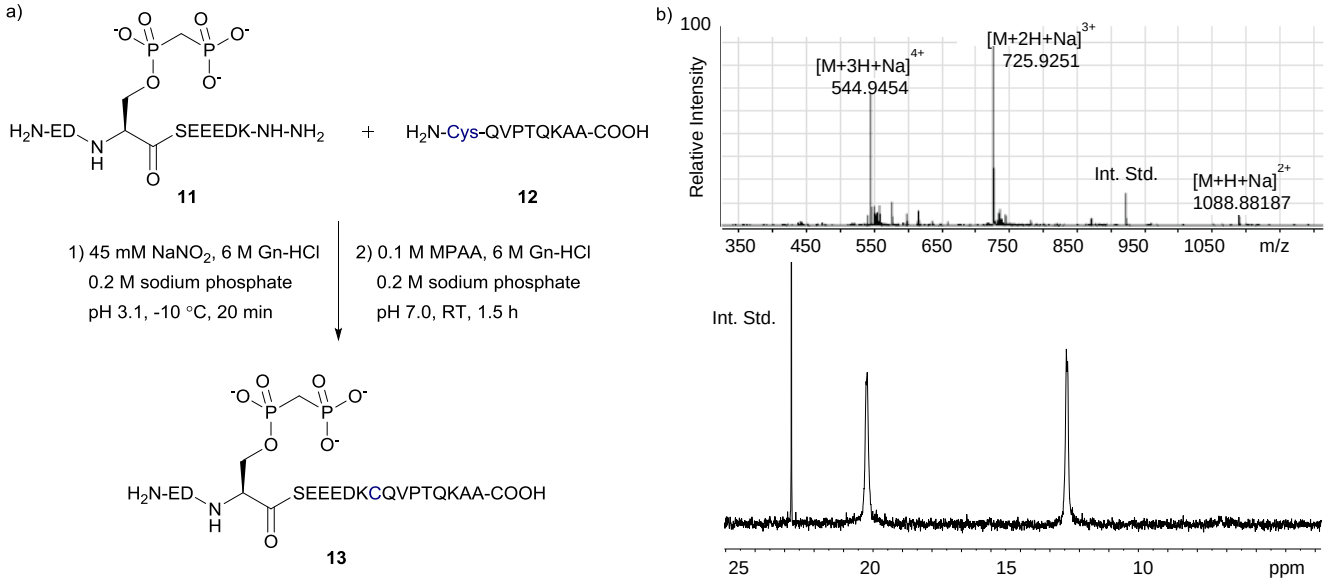


Figure S6. *Characterization of Native Chemical Ligation Product* ***13.*** a) Detailed conditions for ligation between PCP-peptide-hydrazide **11** and *N*-terminal cysteine peptide **12** to yield **13**, a cysteine-containing version of a Nopp140 fragment (amino acids 89-106, $A^{98}C$) b) High-resolution mass spectrometry and ^{31}P NMR at pH 8.23 of peptide **13** confirming the desired product and the integrity of the methylene bisphosphonate moiety. Abbreviations: Gn-HCl = guanidine hydrochloride, MPAA = 4-mercaptophenylacetic acid.

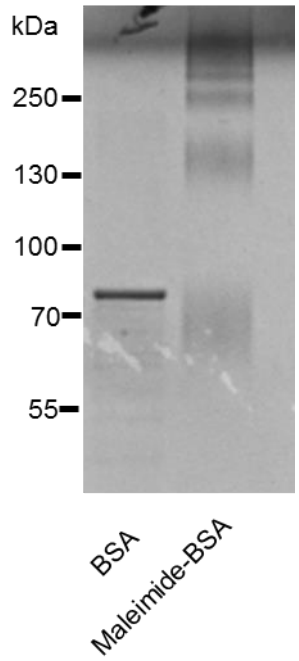


Figure S7. *Comassie G-stained, SDS-PAGE gel of commercial BSA and commercial Maleimide-BSA* Lane 1: Commercially available BSA (Sigma Aldrich) showed a clear, sharp band around 70 kDa, which is the expected molecular weight for this protein. Lane 2: Gel electrophoresis of commercially available maleimide-activated BSA (Sigma Aldrich) exposed multiple broad bands, many of which have molecular weights greater than the expected 67 kDa. BSA contains 35 cysteine residues, which can react with the maleimide functionality prior to any ligation reaction with peptide substrates thus resulting in dimerization and further oligomerization of the BSA monomer proteins. We postulate that the bands are broad since multiple cross-links can form intramolecularly within the BSA monomers, which can impede denaturation of the protein's structure.

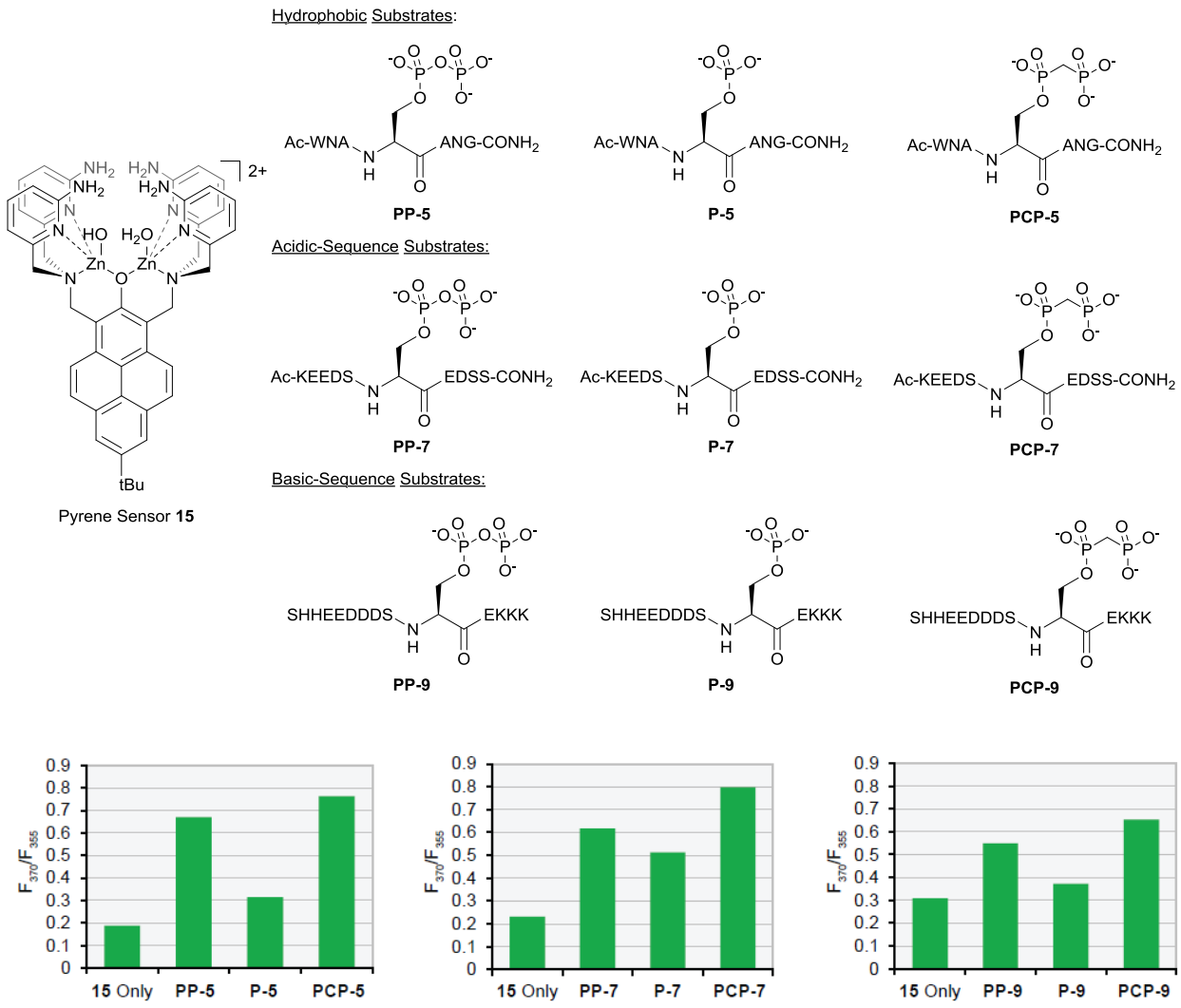


Figure S8. *Fluorescence Measurements of Pyrene Dye* ***15*** *with Peptides Containing Monomer* ***1*** *Compared to Corresponding Pyrophosphopeptides and Phosphopeptides.* Conditions: 3 μM dye **15** with 10 μM analyte in 1:1 DMSO:HEPES (50 mM, pH 7.15) solvent. The fluorescence emission intensity at 438 nm was recorded after excitation at 370 nm and 355 nm. The ratios (F_{370}/F_{355}) of fluorescence emission with the different peptides at 438 nm are shown. Note that acidic peptide sequences tend to bind more favorably to sensor **15** thus explaining the higher ratio observed for peptide **P-7** compared to the other control phosphopeptides (**P-5** and **P-9).**

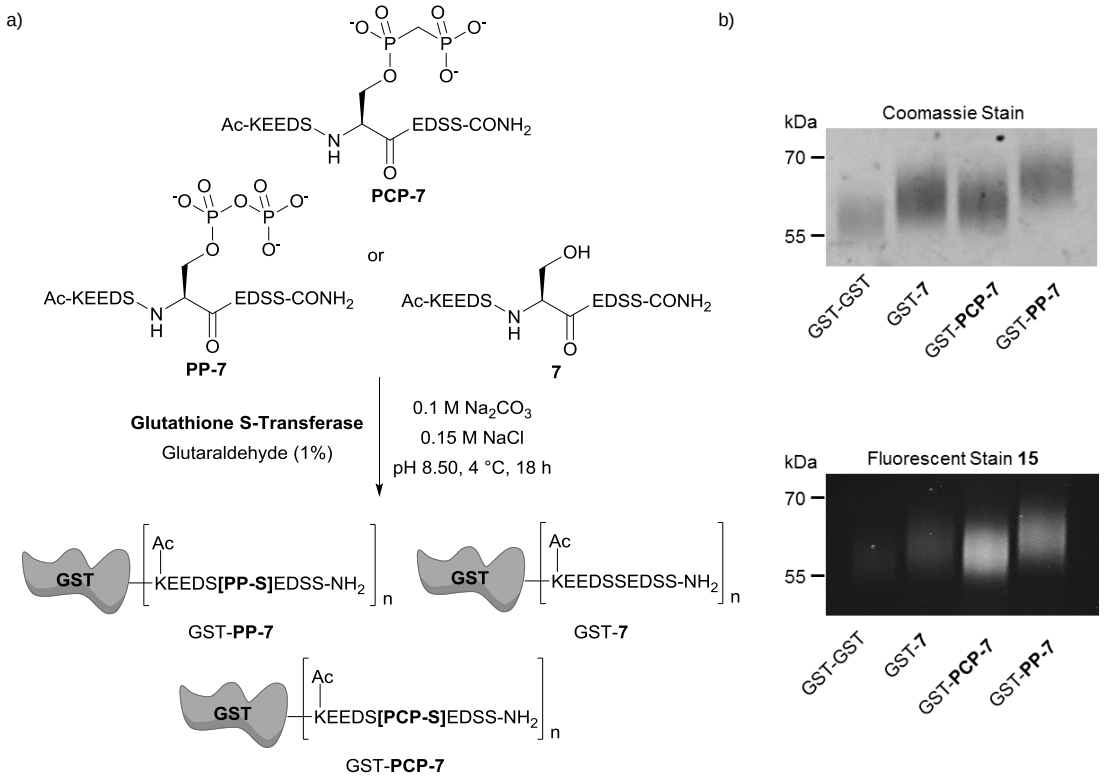
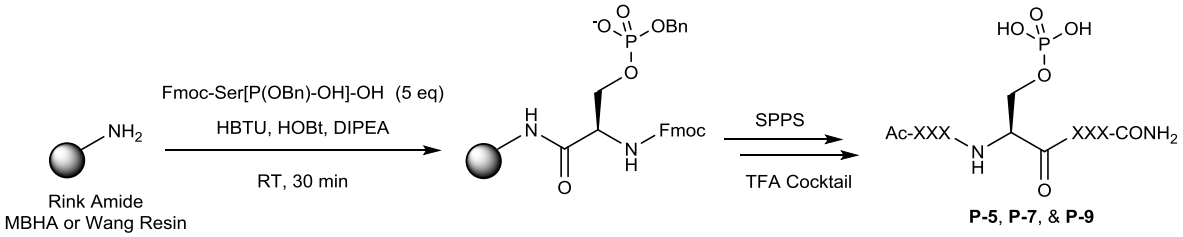


Figure S9. *Glutaraldehyde Ligation of Peptides* ***PCP-7****,* ***PP-7****, and* ***7*** *to GST Protein and Visualization by Gel Stain* ***15***. a) Conditions for glutaraldehyde ligation of **PCP-7**, pyrophosphopeptide **PP-7** and dephosphorylated peptide **7**, which yield ligated GST-peptide products, GST-**PCP-7,** GST-**PP-7**, and GST-**7**. b) Commassie-G staining (top) and fluorescent dye staining with **15** (bottom) of SDS-PAGE gel (10% Bis Tris gel, XT-MES running buffer) show successful ligation and integrity of bisphosphonate moiety, respectively. Note that GST (molecular weight = 26 kDa) is known to self-associate, and upon glutaraldehyde ligation, forms a stable dimer (molecular weight ~52 kDa). The destaining time for this gel was only 4 hours to show the similarity between the GST-**PCP-7** and GST-**PP-7** intensity after fluorescent staining. After overnight destaining of the gel, the background fluorescence intensities of the GST-GST dimer and GST-**7** were negligible, but the intensity of the GST-**PP-7** compared to GST-**PCP-7** had significantly decreased, presumably due to decomposition of the pyrophosphate moiety over time. The gel was visualized at 365 nm excitation for gel fluorescence.

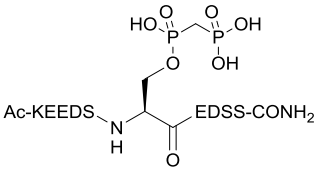
Table S1. *Isolated Yields of Phosphopeptides* ***P-5***, ***P-7***, *and* ***P-9*** *by Solid-Phase Peptide Synthesis (SPPS)*



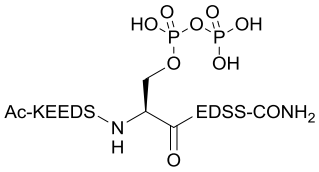
Entry	Peptide Sequence[a]	Sequence Origin	% Isolated Yield[b]
1	Ac-WNASANG-$CONH_2$ (**P-5**)	Random	41
2	Ac-KEEDSSEDSS-$CONH_2$ (**P-7**)	CK2 Consensus	56
3	SHHDDEEESSEKKK (**P-9**)	Rpa34 (Pol1 Subunit)	85[c]

[a]Unless otherwise noted, peptides are *N*-/C-terminally deprotected. Product number listed in parentheses. Red amino acid bares the phosphoryl group. [b]Based on initial resin loading. [c]Significant number of basic amount acids increases TFA-salt adduct formation thus inflating yield based on initial resin loading. Note, for all peptides, TFA Cocktail = 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane, 2.5 % H_2O. Abbreviations: Bn = benzyl; HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt = hydroxybenzotriazole; DIPEA = *N*,*N*-diisopropylethylamine.

Table S2. *Comparative Stability of* ***PCP-7*** *and* ***PP-7*** *in Common TFA Cleavage Cocktails*



PCP-7

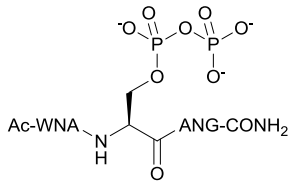


PP-7

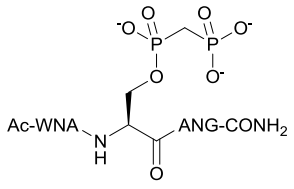
Entry	Substrate	TFA Cocktail[a]	% Hydrolysis[b]
1	PyroP-CK2 Cons. (**PP-7**)	A	52.0
2	PCP-CK2 Cons. (**PCP-7**)	A	2.7
3	PyroP-CK2 Cons. (**PP-7**)	B	26.0
4	PCP-CK2 Cons. (**PCP-7**)	B	0.0

Conditions: 17.5 μM Peptide, TFA Cocktail A or B (1 mL), room temperature, 4 hours. [a]TFA Cocktail A = 95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% H_2O. TFA Cocktail B = 1% trifluoroacetic acid in DCM. [b]Percent hydrolysis measured by analytical HPLC. Normalized to percent hydrolysis at time T = 0 (% hydrolysis = 0% for **PCP-7** and 4.9% for **PP-7** at time T = 0).

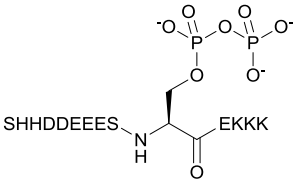
Table S3. *Stability of* ***PCP-5*** *and* ***PCP-9*** *Compared to Corresponding Pyrophosphopeptides* ***PP-5*** *and* ***PP-9*** *in Common Buffers and in the Presence of Divalent Metal Cations*



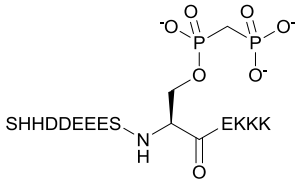
PP-5



PCP-5



PP-9



PCP-9

Entry	Buffer/Metal	pH	% Hydrolysis[a,b] PP-5	% Hydrolysis[a] PCP-5	% Hydrolysis[a] PP-9	% Hydrolysis[a] PCP-9
1	0.1 M HCl	0.89	decomp[c]	0	6.5	0
2	50 mM HEPES	7.10	47	0	21	0
3	50 mM Imidazole	7.23	3.2	0	0	0
4	50 mM Tris	7.52	3.5	0	22	0
5	50 mM MOPS	7.89	26	0	0	0
6	0.1 M NaOH	13.1	decomp[d]	decomp[d]	decomp[d]	decomp[d]
7	200 μM Mg^{2+}	7.89	1.4	0	0	0
8	200 μM Zn^{2+}	7.89	0	0	0	0
9	200 μM Fe^{2+}	7.89	3.4	0	15	0
10	200 μM Mn^{2+}	7.89	0.5	0	0	0
11	200 μM Ca^{2+}	7.89	1.3	0	4.5	0
12	200 μM Co^{2+}	7.89	0	0	0	0
13	200 μM Cu^{2+}	7.89	1.6	0	0	0

Abbreviations: HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, MOPS = 3-(N-morpholino)propanesulfonic acid. Conditions: *Entries 1-6*: 50-100 mM buffer, 100 mM peptide, 37 °C, 24 hours. *Entries 7-13*: 50mM MOPS (pH 7.89), 100 mM peptide, 200 mM divalent metal chloride salt, 37 °C, 24 hours. [a]Percent hydrolysis measured by analytical HPLC. Normalized to time t = 0 min (7.7% for **PP-5**, 0.0% for **PP-9,** 0.0% for **PCP-5** and 0.0% for **PCP-9**). [b]Data previously reported in Yates, L.M. *et. al.* (ref S1).[2] [c]decomp = decomposition though an unknown pathway: Multiple peaks detected with later retention times (i.e. more non-polar productions), which could not be detected by mass spectrometry. [d]decomp = decomposition through a proposed β-elimination pathway.

II. General Information

Commercially available chemicals were purchased from Sigma-Aldrich, Acros Organics, Fisher Scientific, Anaspec and Novabiochem. The following solvents were used: EtOAc = Ethyl acetate (Reagent Grade), DCM = Dichloromethane (Reagent Grade), DMF = *N*,*N*-Dimethylformamide (Reagent Plus®), DMA = *N*,*N*-Dimethylacetamide (Reagent Plus®), Acetone (Reagent Grade), MeCN = Acetonitrile (HPLC Grade), AcOH = Acetic acid (Reagent Grade), Toluene (Reagent Grade), and TFA = Trifluoroacetic Acid (Reagent Grade). Dichloromethane and toluene were dried by passing through activated alumina columns, and DMF was dried by passing through a column of activated molecular sieves[3] using a Pure Process Technology drying system. All reagents were used as received unless otherwise specified.

Thin layer chromatography (TLC) was conducted on EMD Silica Gel 60 F_{254} 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.

Reverse-phase preparative high-performance liquid chromatography (HPLC) was performed on a Varian system with SD-1 prep solvent delivery system, a ProStar 325 UV-Vis detector, and a 440-LC fraction collector, using a Waters XBridge™ 5 μm C18 column (19.0 x 150 mm). Peptides were purified using Solvent A (H_2O with 0.1% TFA) and Solvent B (10% H_2O in MeCN with 0.1% TFA). For the purification of intermediate **4**, Solvent A remained the same, but Solvent B (MeCN with 0.1% TFA) was used. All preparatory HPLC conditions are listed as follows: [column; time point (t) with gradient (in terms of %B in A); flow rate; UV absorbance; retention time of product(s)]. A linear gradient between time points was used unless otherwise specified. For specific HPLC purification methods, see Chemical Synthesis section.

Semi-preparatory HPLC and analytical HPLC were performed on an Agilent 1260 Infinity Quaternary LC system with quaternary solvent delivery system, an autosampler, a diode-array detector, and a fraction collector, using a Vydac 218TP510 5 μm C18 column for semi-preparatory applications or a Vydac 218TP 5 μm C18 column or a Phenomenex Luna™ 3μm C8(2) column for analytical applications. Semi-preparatory purification of phosphopeptides, pyrophosphopeptides and peptides containing analog **1** was performed on a C18 semi-prep column (10 mm x 250 mm) with multiple sample injections. Purity the peptides was assayed on a C18 analytical column (4.60 mm x 150 mm). All chemical, biochemical, and lysate stability studies were monitored by analytical HPLC using the C18 or C8 analytical column. For both semi-preparatory and analytical HPLC applications, Solvent A (H_2O with 0.1% TFA) and Solvent B (10% H_2O in MeCN with 0.1% TFA) were used. All semi-preparatory and analytical HPLC conditions are listed as follows: [column; time point (t) with gradient (in terms of %B in A); flow rate; UV absorbance; retention time of product(s)]. A linear gradient between time points was used unless otherwise specified. See Chemical Synthesis section for detailed methods to isolate the peptides by semi-preparatory methods and to assay purity of individual peptides by analytical HPLC.

^{1}H, ^{13}C, and ^{31}P spectra were recorded on a Bruker 500 AVANCE spectrometer (500, 125, and 202 MHz respectively) with cryocool. NMR data are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, td = triplet of doublets, ddt = doublet

of doublet of triplets, dddd = doublet of doublet of doublet of doublets, m = multiplet), coupling constant (Hz), and integration. Where applicable, ^{31}P spectra were referenced to a tetramethylphosphonium bromide (Me_4PBr) standard (22.77 ppm) in a D_2O capillary insert. All NMR samples of PCP-peptides and pyrophosphopeptides were prepared in H_2O solvent, and 1 M HCl or 1 M NaOH was used to adjust the pH of the samples between pH 7.5-8.5. The internal D_2O capillary was also used to lock the sample.

Optical rotation data for the Fmoc-*L*-serine staring material and for intermediate **2** and analog **1** were obtained on a Perkin Elmer Model 341 Polarimeter. The samples ranged in concentration from 0.400-0.872 g/100 mL, and samples were analyzed in a 1 mL cell volume with a 100 mm path length at 589 nm with a sodium lamp. Each sample was measured in triplicate.

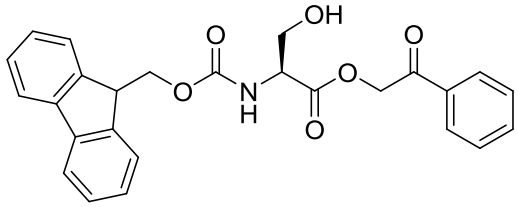
The identity of non-phosphorylated peptides **5**, **7**, **8**, and **12** and phosphopeptides **P-5**, **P-7**, and **P-9** was confirmed by mass spectrometry on a Bruker Daltonics micrOTOF$_Q$ instrument (MSTOF $[M+H]^+$), using 50% H_2O in MeCN with 0.1% formic acid. High resolution mass spectra (HRMS $[M+H]^+$) of PCP-peptides **PCP5-10**, **11**, **13**, and **14**, pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** and monomer **1** and its intermediates were obtained on an Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF) in positive ion mode detection, using 53% H_2O in CH_3CN with 0.05% formic acid as the solvent mixture or in negative ion mode detection, using 50% H_2O in MeCN with 2.5 mM ammonium acetate as the solvent mixture.

Solution-phase fluorescence spectrophotometry was performed using a Fluorolog-3 Spectrofluoremeter (Horiba Jobin Yvon). A 1 nm slit width was used for excitation and emission, and the standard concentrations were 3 μM fluorescent complex **15** and 10 μM analyte (Peptides **PCP-5**, **PP-5**, **P-5**, **PCP-7**, **PP-7**, **P-7**, **PCP-9**, **PP-9**, and **P-9**) in a 1:1 DMSO:HEPES (100 mM, pH 7.15) solution. The fluorescent measurements were taken using a quartz cuvette with 3 mL sample volume. The fluorescence emission intensity at 438 nm was recorded after excitation at 370 nm and 355 nm. The ratios (F_{370}/F_{355}) of fluorescence emission at 438 nm with the different peptides are shown in Figure S6. The fluorescence measurements were run using the FluorEssence program.

Criterion pre-cast polyacrylamide gels (10% Bis-Tris) and XT-MES buffer were obtained from Bio-Rad, and Coomassie-G SafeStain was purchased from Invitrogen. Gel imagining by transillumination was performed on an AlphaImager HP system, with dual UV wavelength (302 nm, 365 nm) functionalities for fluorescent dye stain imaging. Coomasie stain visualization was performed on a GE Healthcare LAS 4000 ImageQuant.

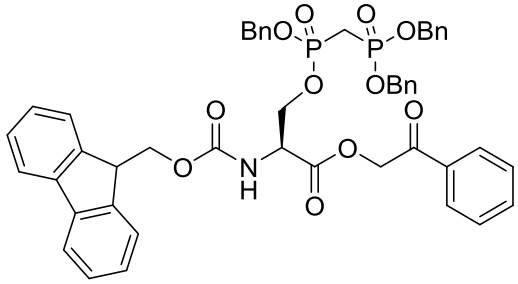
III. Chemical Synthesis

Synthesis of Pyrophosphoserine Analog 1



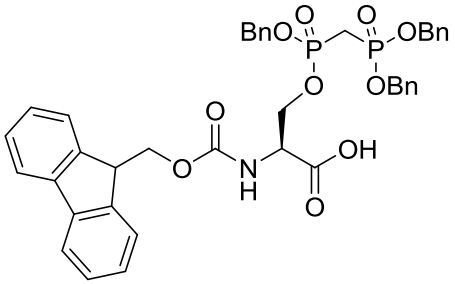
2-oxo-2-phenylethyl (((9*H*-fluoren-9-yl)methoxy)carbonyl)-*L*-serinate (2)

The title compound was synthesized using a modified procedure from Wakamiya, T. et. al.[4] Commerically available fluorenylmethyloxycarbonyl (Fmoc)- *L*-serine (2.00 g, 6.12 mmol, $[\alpha]_D^{20}$ = -0.102 ± 0.001) was dissolved in EtOAc (50.0 mL) at room temperature. To this solution was added dicyclohexylamine (DCHA) (1.22 mL, 6.12 mmol), and the resulting suspension was stirred at room temperature. After stirring for 2 hours, the reaction mixture was filtered to give a quantitative yield of the DCHA salt as a white powder, which was dried under vacuum. After the intermediate was dried for 30 minutes, the DCHA salt (~3.12 g, 6.12 mmol) and phenacyl bromide (1.34 g, 6.73 mmol) were dissolved in DMF (30 mL) and catalytic TEA (86.0 μL, 0.612 mmol) was added to the reaction mixture. After the reaction was stirred for 3.5 hours, the solution was filter to remove the yellow-orange precipitate, and the filtrate was concentrated under vacuum. The resulting oily residue was redissolved in EtOAc (50 mL) with 1 M citric acid (10 mL) and was extracted. The organic layer was washed successively with 1 M citric acid (1 x 10 mL), saturated $NaHCO_3$ (2 x 20 mL), and saturated NaCl (2 x 10mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under vacuum. The crude product was triturated in hexanes (20 mL) to give a tan powder, which was recrystallized from EtOAc (40 mL at 80 ˚C) to give the title compound in 68% yield (1.85 g, 4.15 mmol) as an off-white, crystalline solid. The ^{1}H NMR spectra matched the data previously reported.[3] ^{1}H NMR (500 MHz, DMSO-d6): δ 7.97 (d, *J* = 7.8 Hz, 2H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.75 (dd, *J* = 7.8 Hz, 5.2 Hz, 3H), 7.69 (q, *J* = 10.9 Hz, 9.1 Hz, 1H), 7.56 (t, *J* = 7.7 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 2H), 5.60 – 5.48 (m, 2H), 5.02 (t, *J* = 6.0 Hz, 1H), 4.40 – 4.19 (m, 3H), 3.78 (ddt, *J* = 53.4 Hz, 11.4 Hz, 5.4 Hz, 2H).; ^{13}C NMR (125 MHz, D_2O): δ ^{13}C NMR (125 MHz, DMSO-d6) δ 192.9, 170.8, 156.6, 144.3, 141.2, 134.5, 134.2, 129.4, 128.3, 128.1, 127.6, 125.8, 120.6, 67.4, 66.3, 61.67, 57.3, 47.1; HRMS $[M+H]^+$ calcd for $C_{26}H_{24}NO_6^+$ 446.1598, found 446.1593; Optical Rotation: $[\alpha]_D^{20}$ -0.120 ± 0.001.



2-oxo-2-phenylethyl *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-O-((benzyloxy)((bis(benzyloxy) phosphoryl)methyl)phosphoryl)-*L*-serinate (3)

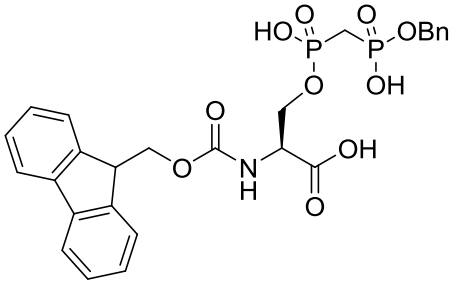
A solution of [bis(benzyloxy)phosphorylmethyl]phosphonic acid monobenzyl ester[5] (2.83 g, 6.30 mmol) in dry toluene (35 mL) was placed under an N_2 atmosphere at 0 ˚C and was treated with drop-wise-addition of oxalyl chloride (984 μL, 11.4 mmol) and catalytic DMF (3 drops). After allowing the solution to warm to room temperature for 2 hours, the reaction was filtered and concentrated under vacuum to yield the crude phosphoryl chloride. A solution of compound **2** (804 mg, 1.80 mmol) in dry DCM (90 mL) was placed in another over-dried flask and was treated with 4-dimethylaminopyridine (988 mg, 8.10 mmol) and 1-*H*-tetrazole (5.40 mL, 0.450 M in MeCN, 1.80 mmol) at -20 ˚C. The crude phosphoryl chloride (2.79 g, 6.00 mmol) in dry DCM (10 mL) was added to the reaction mixture over 10 minutes. After the reaction was allowed to warm to room temperature for 2 hours, the reaction mixture was directly extracted with 50 mM glycine buffer (pH 3.2, 2 x 30 mL) followed by sat. aq. NaCl (1 x 30 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The compound was dry loaded onto silica for flash chromatography (Combiflash, 80 g GOLD column, 0% EtOAc to 100% EtOAc in hexanes) to give the title compound in 61% yield (950 mg, 1.09 mmol) as a clear oil. The purity by ^{1}H NMR was determined to be 95% and the product was carried on to the formation of **3** with no adverse effect to the reaction. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (d, *J* = 7.1 Hz, 1H), 7.80 (d, *J* = 7.3 Hz, 1H), 7.79 – 7.71 (m, 3H), 7.67 (t, *J* = 6.5 Hz, 1H), 7.60 (td, *J* = 7.4 Hz, 3.5 Hz, 1H), 7.46 (q, *J* = 7.5 Hz, 2H), 7.38 – 7.23 (m, 9H), 6.93 (d, *J* = 7.9 Hz, 1H), 5.59 – 4.85 (m, 8H), 4.84 – 4.51 (m, 2H), 4.42 (dddd, *J* = 18.3 Hz, 10.6 Hz, 7.0 Hz, 3.3 Hz, 2H), 4.26 (ddt, *J* = 21.8 Hz, 10.9 Hz, 7.7 Hz, 2H), 2.70 – 2.44 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 191.2, 169.2, 156.9, 144.1 (dd, *J* = 21.4 Hz, 13.9 Hz), 141.4, 135.9 (d, *J* = 6.6 Hz), 134.11 (d, *J* = 2.8 Hz), 134.07, 129.0, 128.8, 128.7 (d, *J* = 7.0 Hz), 128.6, 128.3 (d, *J* = 7.0 Hz), 128.0 – 127.7 (m), 127.2, 125.6 (dd, *J* = 20.4 Hz, 12.6 Hz), 120.8 – 119.5 (m), 68.7 – 68.4 (m), 68.2 (dd, *J* = 6.1 Hz, 3.2 Hz), 67.4, 66.9, 54.6 (dd, *J* = 23.2 Hz, 7.3 Hz), 47.2, 26.0 (t, *J* = 137.1 Hz); ^{31}P NMR (202 MHz, $CDCl_3$) δ 20.6 (dd, *J* = 9.0 Hz, 5.9 Hz, 1P), 20.3 (t, *J* = 4.9 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{48}H_{46}NO_{11}P_2^+$ 874.2541, found 874.2542.



_N_-(((9_H_-fluoren-9-yl)methoxy)carbonyl)-_O_-((benzyloxy)((bis(benzyloxy)phosphoryl)methyl) phosphoryl)-_L_-serine (4)

A solution of compound **3** (950 mg, 1.09 mmol) in 90% AcOH (10 mL) was treated with zinc dust (1.07 g, 16.3 mmol) added in 3 portions over 20 minutes. The heterogeneous reaction was stirred at room temperature and monitored for conversion of starting material by LC/MS. After stirring for 3 hours, the reaction was concentrated under vacuum. The product was resuspended in 50 mM glycine buffer (pH 3.2, 60 mL) with EtOAc (60 mL), and the resulting suspension was filtered to remove the zinc dust prior to extraction. The aqueous layer was washed again with EtOAc (2 x 60 mL). The organic layers were combined, dried over Na_2SO_4, and concentrated under vacuum to give a crude oil. Preparative HPLC purification [C18; t = 0 min 55% of solvent B in solvent A; t = 20 min 85% of solvent B in solvent A;

20 mL/min; 254 nm; T_R = 9.232 min] gave the title peptide in 70% yield (576 mg, 0.762 mmol) as a clear oil. ^{1}H NMR (500 MHz, MeCN-d3) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.69 (dd, *J* = 7.6 Hz, 3.2 Hz, 1H), 7.67 – 7.62 (m, 1H), 7.38 – 7.21 (m, 19H), 5.10 – 4.93 (m, 6H), 4.50 – 4.13 (m, 6H), 2.68 (td, *J* = 21.2 Hz, 5.4 Hz, 2H); ^{13}C NMR (125 MHz, MeCN-d3) δ 171.1, 157.2 (d, *J* = 26.1 Hz), 144.9, 142.0, 137.2 – 136.8 (m), 129.5 – 129.40 (m), 129.35 (d, *J* = 4.7 Hz), 129.0 (d, *J* = 6.2 Hz), 128.6 , 128.0 , 126.2 (d, *J* = 13.0 Hz), 120.9 (d, *J* = 2.6 Hz), 68.9 (ddd, *J* = 29.1 Hz, 11.3 Hz, 6.2 Hz), 67.5, 67.1 (dd, *J* = 36.1 Hz, 5.2 Hz), 55.1 (dd, *J* = 17.6 Hz, 6.1 Hz), 47.8, 25.3 (td, *J* = 135.5 Hz, 8.2 Hz); ^{31}P NMR (202 MHz, MeCN-d3) δ 20.7 (q, *J* = 5.9 Hz, 1P), 20.4 (q, *J* = 6.4 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{40}H_{40}NO_{10}P_2^+$ 756.2122, found 756.2099.



_N_-(((9_H_-fluoren-9-yl)methoxy)carbonyl)-_O_-((((benzyloxy)(hydroxy)phosphoryl)methyl)(hydroxy) phosphoryl)-_L_-serine (1)

A solution of compound **4** (576 mg, 0.762 mmol) in acetone (64.0 mL) was treated with sodium iodide (286 mg, 1.91 mmol). The reaction mixture was heated to reflux (~ 70 – 75 ˚C) and was stirred overnight. After refluxing and stirring for 18 hours, the solution became bright yellow, and the desired product formed as an off-white precipitate. The crude product was collected by vacuum filtration, washed with cold acetone, and dried under vacuum. The solid was redissolved in water (5 mL) and was purified by ion exchange chromatography (Dowex® 50WX8 hydrogen form) to give the acid form of the title compound in 86% yield (378 mg, 0.656 mmol) as a white solid. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.07 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 7.6 Hz, 2H), 7.77 (d, *J* = 7.4 Hz, 2H), 7.46 – 7.16 (m, 9H), 5.01 (d, *J* = 7.3 Hz, 2H), 4.49 – 3.78 (m, 6H), 2.48 – 2.24 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 171.8, 156.6, 144.3, 141.1, 138.6 (d, *J* = 6.9 Hz), 128.7, 128.1, 127.9, 127.8, 127.6 (d, *J* = 4.6 Hz), 126.1 (d, *J* = 7.0 Hz), 120.5, 66.4, 66.3 (d, *J* = 4.5 Hz), 64.6 – 64.5 (m), 55.2 (d, *J* = 6.7 Hz), 47.0, 40.6 – 39.7 (m); ^{31}P NMR (202 MHz, DMSO-d6) δ 18.1 (d, *J* = 7.0 Hz, 1P), 18.0 (d, *J* = 7.0 Hz, 1P). Note, the location of the benzyl (Bn) group was confirmed to be on the β-phosphoryl group using a phosphorus-proton correlation experiment. See NMR Spectra section for spectra. HRMS $[M-H]^-$ calcd for $C_{26}H_{26}NO_{10}P_2^-$ 574.1037, found 574.1039; Optical Rotation: $[\alpha]_D^{20}$ -0.051 ± 0.001.

***Procedure A*: General Procedure for Solid-Phase Peptide Synthesis of Peptides (SPPS)**

The SPPS reactions were conducted in Bio-Rad polypropylene columns at room temperature by agitating with nitrogen using a PEEK switching valve. Fmoc-L-amino acids (AA) were used as starting materials, and the primary coupling and activating agents used for all couplings except those with analog **1** were 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) and hydroxyl-benzotriazole (HOBt), respectively, in DMF. For incorporation of monomer **1**, the coupling agent used

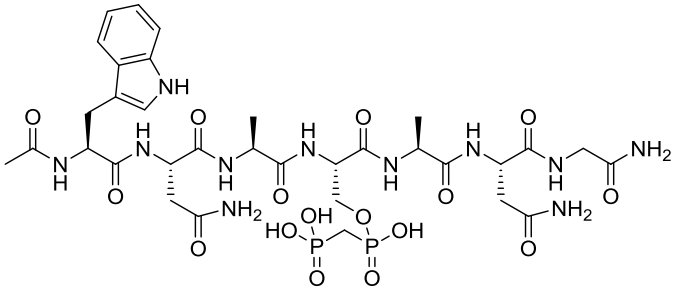
was 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) with no activating agent. Fmoc-Ala-Wang Resin, Fmoc-Lys(Boc)-Wang Resin, Fmoc-Rink-Amide Resin, 2-Chlorotrityl-Chloride Resin, Ninhydrin Test Kit, coupling reagents, activating agents, *N*,*N*-diisopropylethylamine (DIPEA) and Fmoc-L-AA starting materials were purchased from Anaspec, Novabiochem and Sigma Aldrich.

Peptides **PCP-5-10**, phosphopeptides **P-5**, **P-7**, and **P-9**, native chemical ligation substrate **11** and **12**, and dephosphorylated peptides **7** and **8** were prepared by a standard fluorenylmethyloxy-carbonyl (Fmoc) mode solid phase peptide synthesis (SPPS) protocol[6] unless otherwise noted. Briefly, 0.02-0.05 mmol (for PCP-peptides) or 0.05-0.5 mmol (for all other peptides) of resin was swelled for 30 minutes in DMF. Prior to phosphorylated AA incorporation, the resin was treated with 20% piperidine in DMF for 20 minutes to deprotect the Fmoc group. After phosphorylated AA incorporation, the resin was treated with 20% piperidine in DMF for 10 minutes to deprotect the Fmoc group followed by (3 x 1 min) washes with 5% DIPEA prior to coupling to minimize Fmoc-mediated decomposition pathways. For all amino acids except for analog **1**, the resin was incubated with the activated AA (5 eq) in 0.5 M HBTU/HOBt with DIPEA (10 eq) in DMF for 30 minutes with N_2 agitation at room temperature. For incorporation of monomer **1**, the resin was incubated with activated **1** (2.5 eq) in 0.5 M HATU with DIPEA (5 eq) in DMF for 1 hour with N_2 agitation at room temperature. After allowing the suspension to react for 30 minutes to 1 hour, the resin was rinsed with DMF and was dried briefly. A small aliquot of resin was treated with the Ninhydrin Test Kit[7] reagents at 100 ˚C for 6 minutes to monitor for coupling efficiency. If the test result were negative, the cycle was repeated until the desired AA was coupled; if the test result were positive, the resin was incubated with an additional portion of activated AA (5 eq or 2.5 eq for monomer **1**) in 0.5 M HBTU/HOBt solution or in 0.5 M HATU for analog **1**. Upon completion of the AA sequence and a final Fmoc-deprotection step, the full-length peptide was cleaved and globally deprotected with a TFA cocktail [95% TFA, 2.5% triisopropylsilane (TIS), 2.5% H_2O, by volume] for 3 hours to yield the crude peptides. The crude peptides were purified by preparative or semi-preparative HPLC, and the isolated yields reported are based on initial resin loading. All isolated peptides were assayed for purity by analytical HPLC and were analyzed by positive ion mode mass spectrometry to confirm product identity. **PCP-5-10** and **11** were also analyzed by ^{31}P NMR to confirm the integrity of the methylene bisphosphonate moiety.

Procedure B: **General Procedure for Chemical Synthesis of Pyrophosphopeptides**

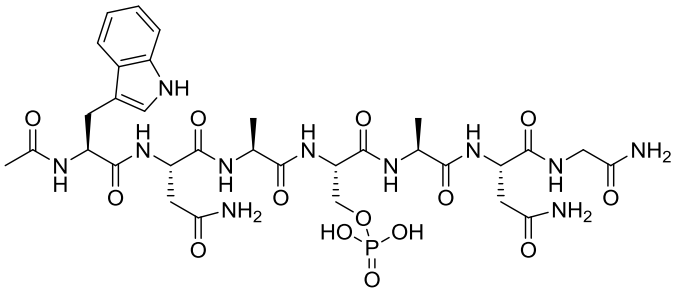
Pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** were synthesized using a modified procedure from Marmelstein, A.M. et. al.[8] The phosphopeptide (4-6.00 μmol) was suspended in DMA and was treated with a solution of lithium benzyl (1*H*-imidazol-1-yl)phosphonate (3 eq, 12-18.0 μmol) in DMA followed by a solution of $ZnCl_2$ (8 eq, 32-48.0 μmol) in H_2O. The overall solvent mixture was 10% H_2O in DMA (4.00 μM). The resulting cloudy white solution was heated to 45 ^oC and stirred at this temperature for 1.5 hours to yield a clear, colorless solution. The reaction mixture was passed through a 0.22 μm syringe filter and directly purified by semi-preparative HPLC. The product fractions were combined and concentrated under vacuum to give a benzyl-protected intermediate as a white, crystalline solid. To a solution of the intermediate (5-7.00 μmol) in 15% H_2O in DMA (3-4.00 μM) was added palladium on carbon (10%, 50% wet, 2 eq, 10-14.0 μmol) followed by triethylamine (4 eq, 20-28.0 μmol). The septum-topped vial was purged with an N_2 atmosphere then purged with H_2 gas. After stirring for 12-15 hours,

the reaction was filtered through a 0.22 μm syringe filter and rinsed with H_2O (100-200 μL). The filtrate containing the product was directly purified by multiple injections on the analytical HPLC unless otherwise noted. The desired product fractions were concentrated under vacuum to give the title compounds as white solids. Yields are reported over two steps. The purity of the isolated peptides was confirmed by analytical HPLC, and the identity of the pyrophosphopeptides was confirmed by mass spectrometry and ^{31}P NMR.



Ac-Trp-Asn-Ala-[PCP-Ser]-Ala-Asn-Gly-CONH$_2$ (PCP-5)

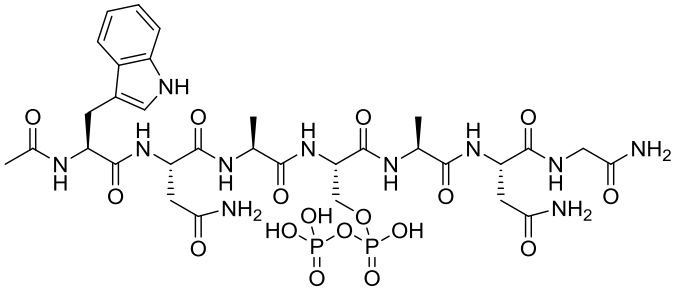
Peptide **PCP-5** was synthesized on an Fmoc-Rink-Amide Resin (22.5 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. The TFA cleavage time was increased to 4 hours to ensure complete deprotection of the benzyl group on the bisphosphonate moiety. Preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 14 min 35% of solvent B in solvent A; 20 mL/min; 280 nm; T_R = 6.262 min] gave the title peptide in 29% yield (5.90 mg, 6.43 μmol) as a white solid. 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; 280 nm; T_R = 8.106 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{33}H_{50}N_{11}O_{16}P_2^+$ 918.2907, found 918.2886; $[M+2H]^{2+}$ calcd for $C_{33}H_{51}N_{11}O_{16}P_2^{2+}$ 459.6490, found 459.6497) and ^{31}P NMR (202 MHz, H_2O, pH = 8.21) δ 12.83, 20.18.



Ac-Trp-Asn-Ala-[PSer]-Ala-Asn-Gly-CONH$_2$ (P-5)

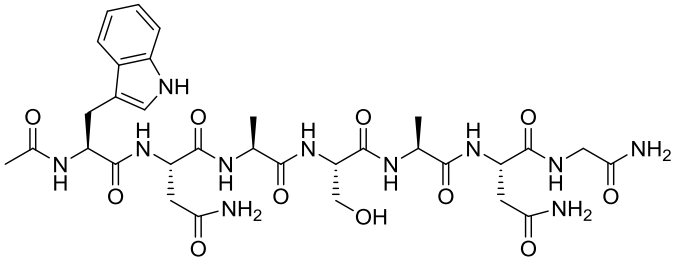
Peptide **P-5** was synthesized on an Fmoc-Rink-Amide Resin (203 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A) as described previously in Marmelstein, A.M. et. al.[8] Preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 14 min 35% of solvent B in solvent A; 20 mL/min; 280 nm; T_R = 7.672 min] gave the title peptide in 41% yield (60.5 mg, 72.0 μmol) as a white solid. The spectral data for **P-5** matched the data reported.[8] 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; 280 nm; T_R = 8.743 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+H]^+$ calcd for $C_{32}H_{47}N_{11}O_{14}P^+$ 840.3036, found 840.2981).



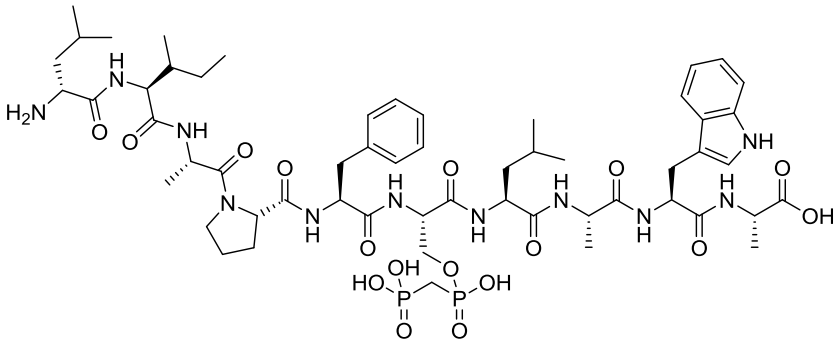
Ac-Trp-Asn-Ala-[PyroP-Ser]-Ala-Asn-Gly-CONH$_2$ (PP-5)

Using Procedure B, pyrophosphopeptide **PP-5** was prepared from phosphopeptide **P-5** (10.0 mg, 11.9 μmol) in 2 steps as described previously.[8] The benzyl-protected intermediate **PP-5-Bn** was purified by analytical HPLC [C18; t = 0 min 5% of solvent B in solvent A, t = 5 min 0% of solvent B in solvent A; t = 20 min 40% of solvent B in solvent A; 1 mL/min; 280 nm; T_R = 16.465 min]. The product of the hydrogenolysis reaction of **PP-5-Bn** was directly purified by analytical HPLC purification [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 30% of solvent B in solvent A; 1 mL/min; 280 nm; T_R = 11.324 min] gave the title peptide in 58% yield (7.00 mg, 7.61μmol) over two steps as a white solid. The spectral data for **PP-5** matched the data reported previously.[8] 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; 280 nm; T_R = 13.389 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+Na]^+$ calcd for $C_{30}H_{45}N_{11}NaO_{16}P_2^+$ 944.2586, found 944.2251; $[M+H+Na]^{2+}$ calcd for $C_{30}H_{46}N_{11}NaO_{16}P_2^{2+}$ 472.6330, found 471.6164) and ^{31}P NMR (202 MHz, D_2O, pD = 7.47) δ -10.98 (d, $^2J_{P\text{-}P}$ = 19.4 Hz), -11.81 (d, $^2J_{P\text{-}P}$ = 19.2 Hz).



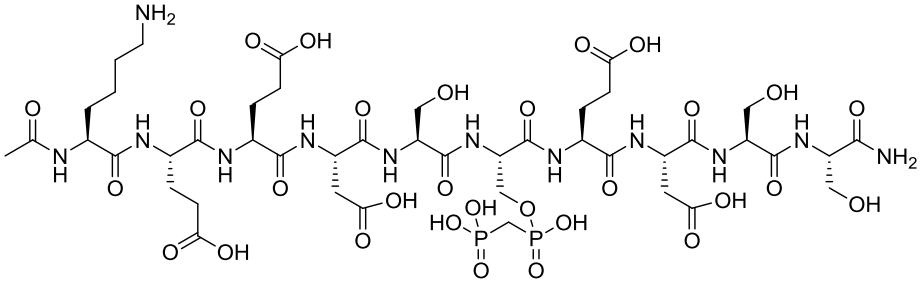
Ac-Trp-Asn-Ala-Ser-Ala-Asn-Gly-CONH$_2$ (5)

The synthesis of compound **5** was reported previously.[8] All spectral data matched that which was previously reported. Compound **5** was used as an analytical standard for assay development for the cell lysate hydrolysis studies (See Assays for Chemical and Lysate Stability Studies of Peptides Containing Analog Section).



H_2N-Leu-Ile-Ala-Pro-Phe-[PCP-Ser]-Leu-Ala-Trp-Ala-COOH (PCP-6)

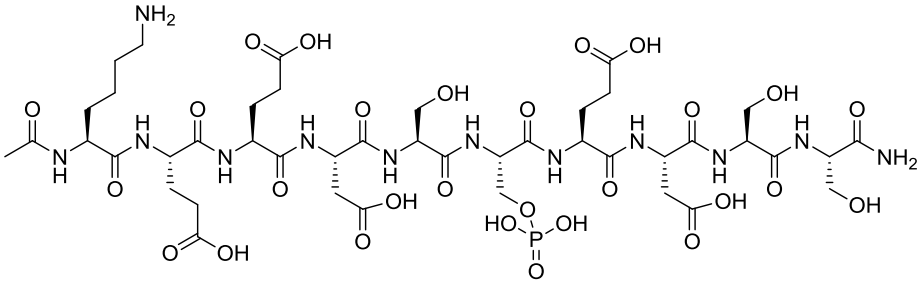
Peptide **PCP-6** was synthesized on an Fmoc-Ala-Wang Resin (25.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). After a singular 1 hour coupling of monomer **1** (2.5 eq), the Ninhydrin Test indicated ~90% conversion. To prevent elongation of truncated products, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (1x10min) to cap any unreacted free amine remaining on the peptide chain. Semi-preparative HPLC purification [C18; t = 0 min 25% of solvent B in solvent A; t = 13 min 55% of solvent B in solvent A; 4 mL/min; 280 nm; T_R = 11.036 min] gave the title peptide in 28% yield (8.70 mg, 6.98 μmol) as a white solid. 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 60% of solvent B in solvent A; 1 mL/min; 280 nm; T_R = 14.807 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{56}H_{86}N_{11}O_{17}P_2^+$ 1246.5673, found 1246.5789; $[M+2H]^{2+}$ calcd for $C_{56}H_{87}N_{11}O_{17}P_2^{2+}$ 623.7873, found 623.7939) and ^{31}P NMR (202 MHz, H_2O, pH = 8.19) δ 19.99, 13.31 (d, $^2J_{P\text{-}P}$ = 8.9 Hz, 1P).



Ac-Lys-Glu-Glu-Asp-Ser-[PCP-Ser]-Glu-Asp-Ser-Ser-$CONH_2$ (PCP-7)

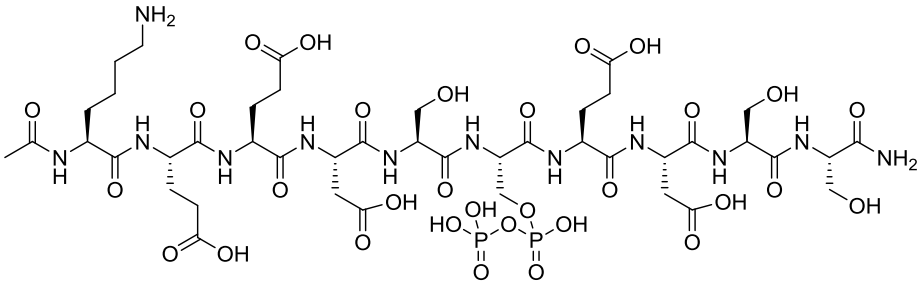
Peptide **PCP-7** was synthesized on an Fmoc-Rink-Amide Resin (33.3 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). Note, for this peptide sequence, a double-coupling (1 hour each) of monomer **1** (2.5 eq) with HATU (2.4 eq) and DIPEA (5 eq) was required for maximum coupling efficiency. After monomer **1** incorporation, HATU (4.9 eq) was used as the coupling reagent for all subsequent AA (5 eq) coupling reactions to improve the efficacy of the synthesis. After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. The TFA cleavage time was increased to 4 hours to ensure complete deprotection of the benzyl group on the bisphosphonate moiety. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 4.905 min] gave the title peptide in 45% yield (19.3 mg, 14.7 μmol) as a white

solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 7.637 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{44}H_{73}N_{12}O_{30}P_2^+$ 1311.4025, found 1311.3927; $[M+2H]^{2+}$ calcd for $C_{44}H_{74}N_{12}O_{30}P_2^{2+}$ 656.2049, found 656.2002) and ^{31}P NMR (202 MHz, H_2O, pH = 8.13) δ 13.93, 19.29.



Ac-Lys-Glu-Glu-Asp-Ser-[PSer]-Glu-Asp-Ser-Ser-CONH$_2$ (P-7)

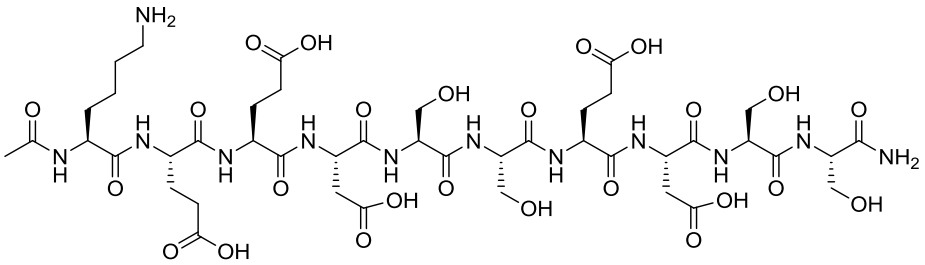
Peptide **P-7** was synthesized on an Fmoc-Rink-Amide Resin (200 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. The TFA cleavage time was increased to 4 hours to ensure complete deprotection of the benzyl group on the bisphosphonate moiety. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 10 min 10% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 4.845-5.152 min] gave the title peptide in 56% yield (45.8 mg, 37.1 μmol) as a white solid. The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 10 min 15% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 7.568 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+H]^+$calcd for $C_{43}H_{70}N_{12}O_{28}P^+$ 1233.4155, found 1233.4264; $[M+2H]^{2+}$ calcd for $C_{43}H_{71}N_{12}O_{28}P^{2+}$ 617.2114, found 617.1852).



Ac-Lys-Glu-Glu-Asp-Ser-[PyroPSer]-Glu-Asp-Ser-Ser-CONH$_2$ (PP-7)

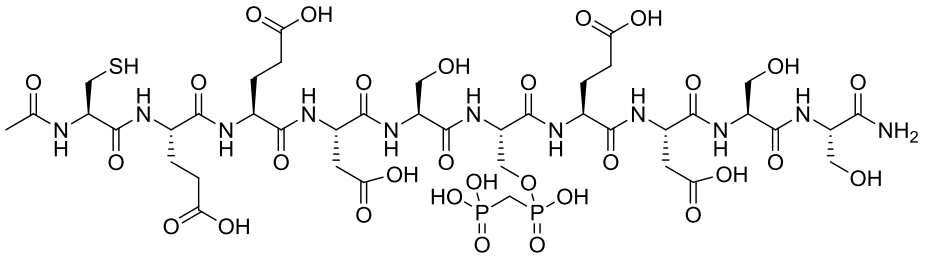
Using a modified version of Procedure B, pyrophosphopeptide **PP-7** was prepared from phosphopeptide **P-7** (12.0 mg, 9.73 μmol) in 2 steps. The benzyl-protected intermediate **PP-7-Bn** was purified by semi-preparatory HPLC [C18; t = 0 min 3% of solvent B in solvent A, t = 6 min 3% of solvent B in solvent A; t = 15 min 20% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 13.854 min]. Due to the strong polarity of intermediate **PP-7-Bn**, a trifluoroacetic acid-mediated removal of the benzyl group was used instead of the hydrogenolysis reaction which proceeds in mostly organic solvents from which desired product **PP-7**

could not be isolated. Intermediate **PP-7-Bn** was treated with a TFA-cocktail (95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% H_2O, by volume) for 1.75 hours at room temperature. Upon reaction completion, the crude product was isolated by cold ether precipitation and centrifuged at 4500 rpm for 15 minutes at 4 ˚C to precipitate the product. The peptide was purified by semi-preparative HPLC [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 4.289 min] to give the title compound in 41% yield (3.30 mg, 2.51 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 = 7.401 min], and the identity of the pyrophosphopeptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{43}H_{71}N_{12}O_{31}P_2^+$ 1313.3818, found 1313.3858; $[M+2H]^{2+}$ calcd for $C_{43}H_{72}N_{12}O_{31}P_2^{2+}$ 657.1945, found 657.1977) and ^{31}P NMR (202 MHz, H_2O, pH = 8.12) δ -6.69 , -10.96 (d, $^2J_{P\text{-}P}$ = 21.7 Hz).



Ac-Lys-Glu-Glu-Asp-Ser-Ser-Glu-Asp-Ser-Ser-CONH$_2$ (7)

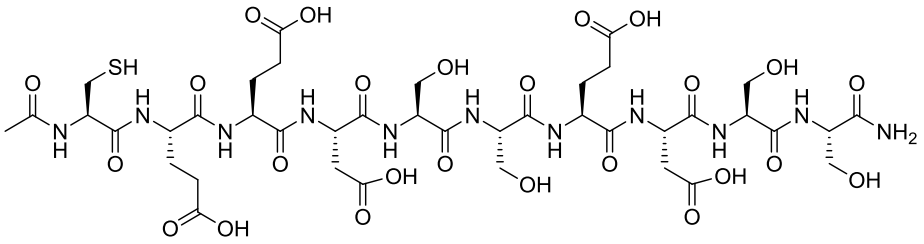
Peptide **7** was synthesized on an Fmoc-Rink-Amide Resin (33.3 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). HATU (4.9 eq) was used as the coupling reagent for all amino acid (5 eq) coupling reactions to improve the efficacy of the synthesis. After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 7.208 min] gave the title peptide in 32% yield (14.5 mg, 12.6 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 8.376 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+H]^+$ calcd for $C_{43}H_{69}N_{12}O_{25}^+$ 1153.4491, found 1153.4514; $[M+2H]^{2+}$ calcd for $C_{43}H_{70}N_{12}O_{25}^{2+}$ 577.2282, found 577.2272).



Ac-Cys-Glu-Glu-Asp-Ser-[PCP-Ser]-Glu-Asp-Ser-Ser-CONH$_2$ (PCP-8)

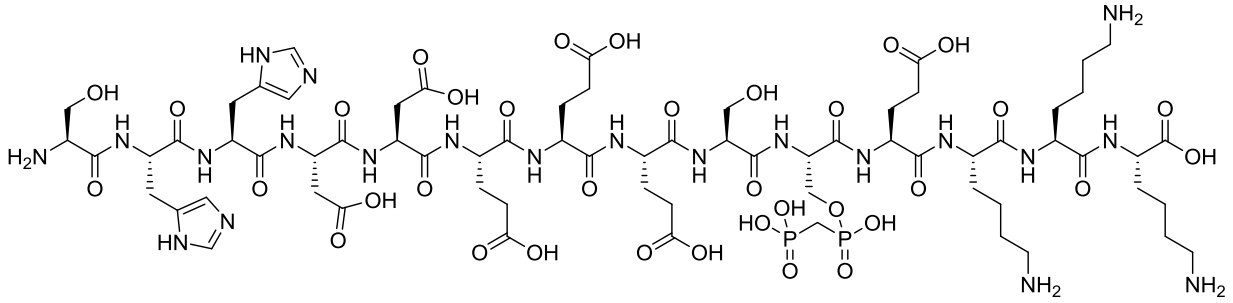
Peptide **PCP-8** was synthesized on an Fmoc-Rink-Amide Resin (20.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). After monomer **1** incorporation, HATU (4.9 eq) was

used as the coupling reagent for all subsequent AA (5 eq) coupling reactions to improve the efficacy of the synthesis. After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. The TFA cleavage cocktail used to cleave and globally deprotect peptide **PCP-8** was 92.5% TFA, 2.5% triisopropylsilane, 2.5% H_2O, and 2.5% ethanedithiol to prevent disulfide formation during the cleavage reaction. The TFA cleavage time was increased to 4 hours to ensure complete deprotection of the benzyl group on the bisphosphonate moiety. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 10% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 7.470 min] gave the title peptide in 33% yield (8.7 mg, 6.77 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 8.498 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{41}H_{66}N_{11}O_{30}P_2S^+$ 1286.3167, found 1286.3185; $[M+2H]^{2+}$ calcd for $C_{41}H_{67}N_{11}O_{30}P_2S^{2+}$ 643.6620, found 643.6627) and ^{31}P NMR (202 MHz, H_2O, pH = 8.11) δ 14.09, 19.17.



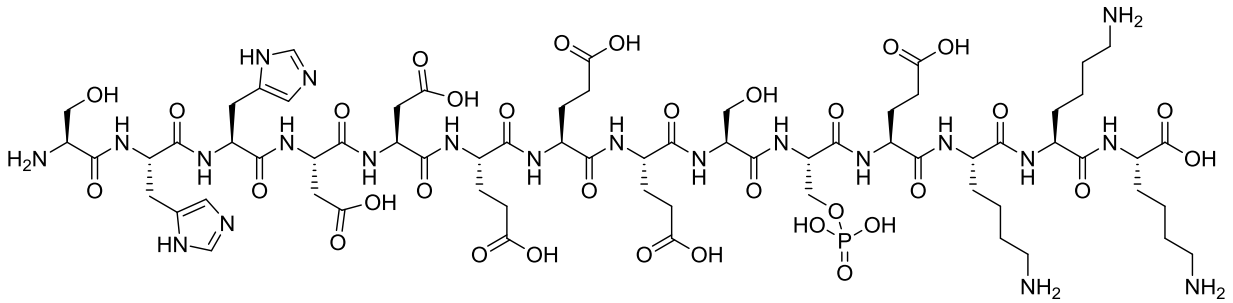
Ac-Cys-Glu-Glu-Asp-Ser-Ser-Glu-Asp-Ser-Ser-$CONH_2$ (8)

Peptide **8** was synthesized on an Fmoc-Rink-Amide Resin (40.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). HATU (4.9 eq) was used as the coupling reagent for all amino acid (5 eq) coupling reactions to improve the efficacy of the synthesis. After the final Fmoc-deprotection step, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (3x10min) to cap the *N*-terminus. The TFA cleavage cocktail used to cleave and globally deprotect peptide **8** was 92.5% TFA, 2.5% triisopropylsilane, 2.5% H_2O, and 2.5% ethanedithiol to prevent disulfide formation during the cleavage reaction. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 9.664 min] gave the title peptide in 33% yield (14.9 mg, 13.2 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 9.670 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+H]^+$ calcd for $C_{40}H_{62}N_{11}O_{25}S^+$ 1128.3634, found 1128.3679; $[M+2H]^{2+}$ calcd for $C_{40}H_{63}N_{11}O_{25}S^{2+}$ 564.6853, found 564.6833).



Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PCP-Ser]-Glu-Lys-Lys-Lys (PCP-9)

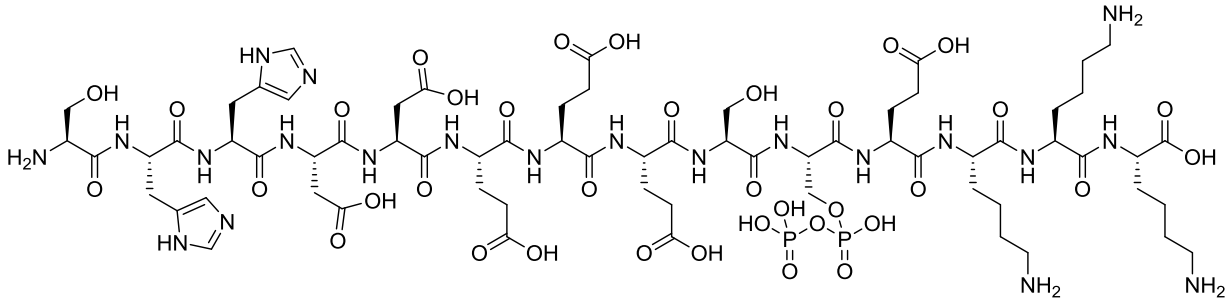
Peptide **PCP-9** was synthesized on an Fmoc-Lys(Boc)-Wang Resin (20.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). All AA coupling reactions for this peptide used HATU (4.9 eq) as the coupling reagent due to the repeat stretches of the same or similar amino acids decreasing the coupling efficiency. After a singular 1 hour coupling of monomer **1** (2.5 eq), the Ninhydrin Test indicated significant free amine still present; as a result, a double-coupling of analog **1** (2.5 eq) with HATU (2.4 eq) and DIPEA (5 eq) for 1 additional hour was required for maximum coupling efficiency. To prevent elongation of truncated products, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (1x10min) to cap any unreacted free amine remaining on the peptide after the second monomer **1** coupling reaction. After analog **1** was incorporated, the coupling efficiency using the standard conditions decreased, presumably due to formation of secondary structure; as a result, all amino acids post-analog **1** incorporation were double-coupled using standard reaction conditions to achieve full conversion. Semi-preparative HPLC purification [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 = 8.191 min] gave the title peptide in 94% yield (34.8 mg, 18.9 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 8.875 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{68}H_{111}N_{21}O_{35}P_2^{2+}$ 921.8508, found 921.8659; $[M+3H]^{3+}$ calcd for $C_{68}H_{112}N_{21}O_{35}P_2^{3+}$ 614.9029, found 614.9123; $[M+4H]^{4+}$ calcd for $C_{68}H_{113}N_{21}O_{35}P_2^{4+}$ 461.4290, found 461.4342) and ^{31}P NMR (202 MHz, H_2O, pH = 8.10) δ 19.95 (d, $^2J_{P\text{-}P}$ = 9.7 Hz, 1P), 13.26 (d, $^2J_{P\text{-}P}$ = 9.9 Hz, 1P).



Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PSer]-Glu-Lys-Lys-Lys (P-9)

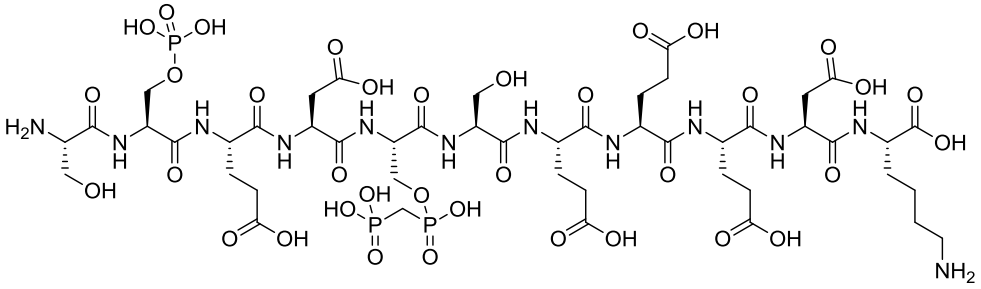
Peptide **P-9** was synthesized on an Fmoc-Lys(Boc)-Wang Resin (45.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). Preparative HPLC purification [C18; t = 0 min 5% of

solvent B in solvent A; t = 10 min 15% of solvent B in solvent A; 20 mL/min; 214 nm; T_R = 3.590 min] gave the title peptide in 85% yield (67.5 mg, 38.3 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 9.150 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+2H]^{2+}$calcd for $C_{67}H_{108}N_{21}O_{33}P^{2+}$ 882.8573, found 882.8563; $[M+3H]^{3+}$ calcd for $C_{67}H_{109}N_{21}O_{33}P^{3+}$ 588.9073, found 588.9082; $[M+4H]^{4+}$ calcd for $C_{67}H_{110}N_{21}O_{33}P^{4+}$ 441.9323, found 441.9361).



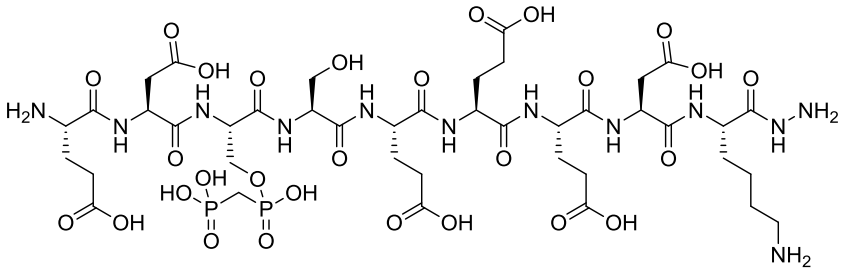
Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PyroP-Ser]-Glu-Lys-Lys-Lys (PP-9)

Using Procedure B, pyrophosphopeptide **PP-9** was prepared from phosphopeptide **P-9** (11.5 mg, 6.52 μmol) in 2 steps. The benzyl-protected intermediate **PP-9-Bn** was purified by preparatory HPLC [C18; t = 0 min 3% of solvent B in solvent A, t = 5 min 3% of solvent B in solvent A; t = 25 min 20% of solvent B in solvent A; 20 mL/min; 214 nm; T_R = 14.187 min). The hydrogenolysis was performed in 35% H_2O in NMF. The resulting filtrate was directly purified by multiple injections on the analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 5 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 = 12.213 min] gave 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 = 9.342 min], and the identity of the peptide 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).



Ser-[PSer]-Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys (PCP-10)

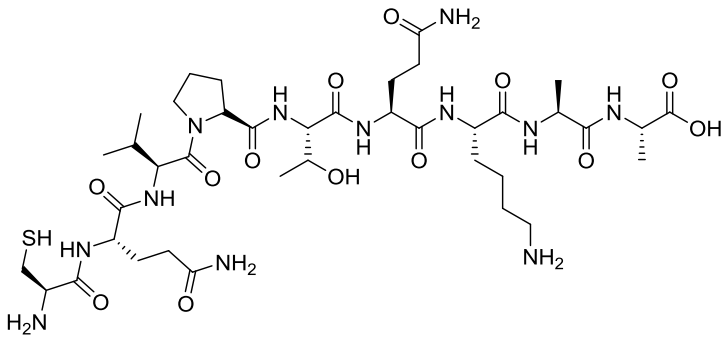
Peptide **PCP-10** was synthesized on an Fmoc-Lys(Boc)-Wang Resin (36.0 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). All AA coupling reactions for this peptide used HATU (4.9 eq) as the coupling reagent due to the repeat stretches of the same or similar amino acids decreasing the coupling efficiency. After a singular 1 hour coupling of monomer **1** (2.5 eq), the Ninhydrin Test indicated significant free amine still present; as a result, a double-coupling of analog **1** (2.5 eq) with HATU (2.4 eq) and DIPEA (5 eq) for 1 additional hour was required for maximum coupling efficiency. To prevent elongation of truncated products, the resin was treated with acetic anhydride (Ac_2O) (20.0 eq) and DIPEA (40.0 eq) in DMF (1x10min) to cap any unreacted free amine remaining on the peptide chain after the second monomer **1** coupling reaction. Analytical HPLC purification [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 = 8.107 min] gave the title peptide in 31% yield (16.2 mg, 11.0 μmol) as a white solid. The purity of the isolated peptide was confirmed by analytical HPLC [C18; t = 0 min 0% of solvent B in solvent A; t = 10 min 15% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 7.821 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd for $C_{47}H_{78}N_{12}O_{36}P_3^+$ 1479.3849, found 1479.3925; $[M+2H]^{2+}$ calcd for $C_{47}H_{79}N_{12}O_{36}P_3^{2+}$ 740.1961, found 740.1984) and ^{31}P NMR (202 MHz, H_2O, pH = 8.12) δ 19.24, 14.19 (d, $^2J_{P\text{-}P}$ = 9.3 Hz, 1P), 3.90.



Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-NH-NH_2 (11)

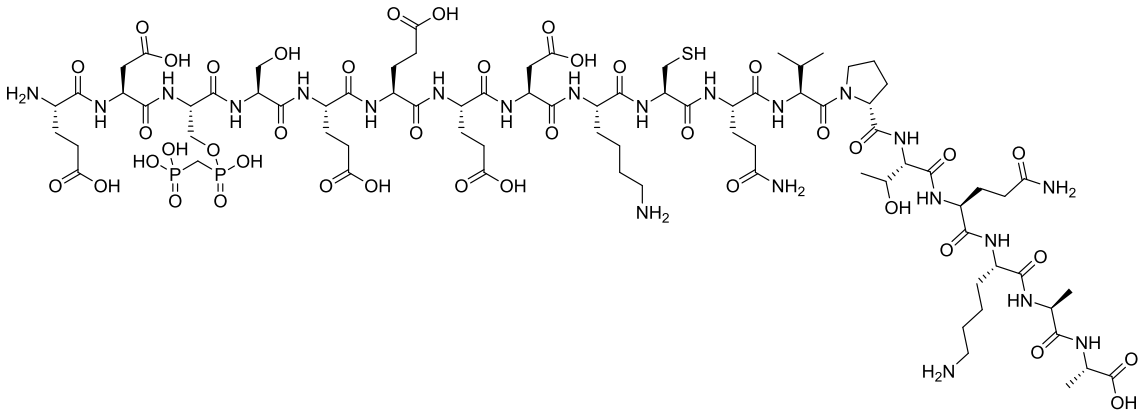
Peptide **11** was synthesized on 2-chlorotrityl-chloride resin (22.4 μmol scale), which was converted in to a hydrazide resin using a modified procedure from Zheng, *et. al.*[9] Briefly, the 2-chlorotritryl-chloride resin was washed with DMF (3x1 mL), followed by DCM (3x1 mL), and finally DMF (3x1 mL) for 10 seconds each. The resin was then swelled with 1:1 DMF:DCM (1 mL total) for 30 minutes with N_2 agitation. After draining the DCM:DMF solution, the resin was agitated with 5% (vol/vol) NH_2NH_2 (1 mL) for 30 minutes at room temperature. The resin was drained, rinsed with DMF, and incubated with another 1 mL portion of 5% NH_2NH_2 for 30 minutes. To cap any unreacted 2-chlorotrityl-chloride resin, 5% (vol/vol) MeOH/DMF was added to the resin, and the sample was agitated with N_2 for 10 minutes. After hydrazination of the resin, the standard Fmoc-mode coupling protocol (See Procedure A) was followed. All amino acid coupling reactions for this peptide used HATU (4.9 eq) as the coupling reagent due to the repeat stretches of the same or similar amino acids decreasing the coupling efficiency and since many of the amino acids had bulky side chains and protecting groups which could decrease the coupling efficiency. The traditional TFA cleavage reaction was performed to yield the crude product. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 15% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 4.924 min] gave the title peptide in 24% yield (6.00 mg, 4.84 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 7.718 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+H]^+$ calcd

for $C_{41}H_{69}N_{12}O_{28}P_2^+$ 1239.3814, found 1239.3863; $[M+2H]^{2+}$ calcd for $C_{41}H_{70}N_{12}O_{28}P_2^{2+}$ 620.1943, found 620.1978) and ^{31}P NMR (202 MHz, H_2O, pH = 8.28) δ 20.33, 13.29 (d, $^2J_{P\text{-}P}$ = 6.1 Hz, 1P).



Cys-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (12)

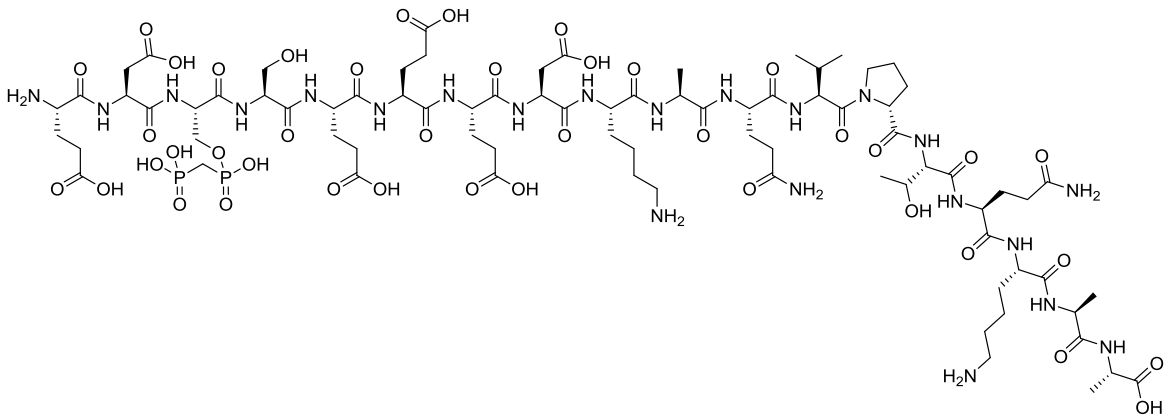
Peptide **12** was synthesized on an Fmoc-Ala-Wang Resin (100 μmol scale) using the standard Fmoc-mode coupling protocol (See Procedure A). Since the bulky valine reside followed the proline residue, for which coupling cannot be monitored by the ninhydrin test, the valine residue was double coupled with HATU (4.9 eq) as the activating agent for 45 minutes per coupling with N_2 agitation. The TFA cleavage cocktail used to cleave and globally deprotect peptide **12** was 92.5% TFA, 2.5% triisopropylsilane, 2.5% H_2O, and 2.5% ethanedithiol to prevent disulfide formation during the cleavage reaction. Semi-preparative HPLC purification [C18; t = 0 min 3% of solvent B in solvent A; t = 13 min 20% of solvent B in solvent A; 4 mL/min; 214 nm; T_R = 12.705 min] gave the title peptide in 63% yield (59.4 mg, 62.9 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 12.125 min], and the identity of the peptide was confirmed by mass spectrometry (MSTOF $[M+H]^+$ calcd for $C_{39}H_{69}N_{12}O_{13}S^+$ 945.4822, found 945.4445; $[M+2H]^{2+}$ calcd for $C_{39}H_{70}N_{12}O_{13}S^{2+}$ 473.2448, found 473.1716).



Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-Cys-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (13)

Peptide **13** was synthesized *via* native chemical ligation (NCL) following a procedure adapted from Zheng, *et. al.*[9] and Brown, *et. al.*[10] utilizing PCP-peptide-hydrazide **11** and cysteine-containing peptide **12** as the starting materials. To convert the acyl-hydrazide into the thioester peptide required for NCL,

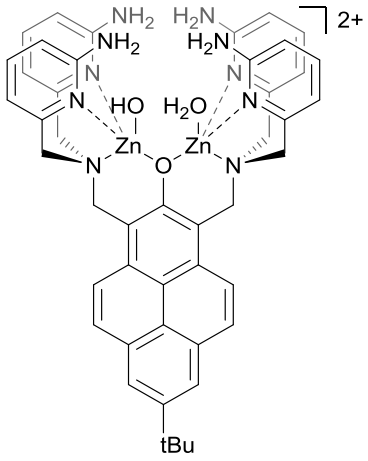
peptide acyl-hydrazide **11** (2.80 mg, 2.26 μmol) was dissolved in the oxidation buffer (45.0 mM $NaNO_2$, 6 M guanidine hydrochloride (Gn-HCl), 200 mM sodium phosphate, pH 3.07, 400 μL total volume) and allowed to react for 20 minutes at -10 ˚C with stirring. After confirming the formation of the acyl-azide intermediate by analytical HPLC and mass spectrometry, the *in situ* thiolysis was performed at room temperature by adding an equal volume (relative to the oxidation, 400 μL total volume) of 6 M Gn-HCl, 200 mM sodium phosphate (pH 3.07) and 4-mercaptophenylacetic acid (13.6 mg, 79.7 μmol) to the reaction tube followed by adjusting the pH to 6.93. After incubating the thiolysis reaction for 15 minutes at room temperature, the reaction mixture was added to solid cysteine-containing-peptide **12** (2.10 mg, 2.22 μmol). The pH was adjusted to 6.96, and the reaction proceeded at room temperature for 2 hours with monitoring by analytical HPLC for conversion of starting materials. Upon complete conversion of the thioester peptide, 33.3 mM of *tris*(2-carboxyethyl)phosphine (TCEP) was added to the reaction to ensure complete reduction of thiols prior to analysis by analytical HPLC and product isolation. Analytical HPLC purification [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 = 13.916 min] initially gave the title peptide in 71% yield (3.50 mg, 1.63 μmol), which was determined to only be 46% pure by analytical HPLC. The primary impurities were residual TCEP and Gn-HCl; no peptidic byproduct remained. The isolated material was repurified using the same gradient as before but with smaller injection volumes to improve separation. The second analytical HPLC purification gave the pure title peptide in 29% yield (1.40 mg, 0.651 μmol). 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 13.773 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+Na+H]^{2+}$ calcd for $C_{80}H_{133}N_{22}NaO_{41}P_2S^{2+}$ 1088.4074, found 1088.8830; $[M+Na+2H]^{3+}$ calcd for $C_{80}H_{134}N_{22}NaO_{41}P_2S^{3+}$ 725.2718, found 725.9252; $[M+Na+3H]^{4+}$ calcd for $C_{80}H_{135}N_{22}NaO_{41}P_2S^{4+}$ 544.2057, found 544.9459) and ^{31}P NMR (202 MHz, H_2O, pH = 8.23) δ 20.22 (d, $^2J_{P\text{-}P}$ = 8.1 Hz, 1P), 13.42 (d, $^2J_{P\text{-}P}$ = 8.8 Hz, 1P).



Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-Ala-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (14)

Peptide **14** was synthesized *via* free radical peptide desulfurization following a procedure adapted from Zheng, *et. al*,[9] Geiermann, *et. al.*[11] and Haase, *et. al.*[12] utilizing NCL product **13** as the starting material. Peptide **13** (1.2 mg, 0.558 μmol) was dissolved in the reaction buffer (200 mM sodium phosphate, 6 M guanidine hydrochloride, 50 mM V-50 [2,2'-Azobis(2-methylpropionamidine)dihydrochloride], 40 mM glutathione reduced, 250 mM TCEP, pH 7.38, 400 μL total volume). The reaction mixture was heated to

37 ˚C with stirring. After incubating the reaction for 3.5 hours, analytical HPLC analysis indicated full conversion to the desired desulfurization product. Analytical HPLC purification [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 = 13.198 min] gave the title peptide in 75% yield (0.900 mg, 0.425 μmol) as a white solid. 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 30% of solvent B in solvent A; 1 mL/min; 214 nm; T_R = 13.023 min], and the identity of the peptide was confirmed by mass spectrometry (HRMS $[M+2H]^{2+}$ calcd for $C_{80}H_{134}N_{22}O_{41}P_2^{2+}$ 1060.9287, found 1060.9202; $[M+Na+2H]^{3+}$ calcd for $C_{80}H_{134}N_{22}NaO_{41}P_2^{3+}$ 714.9489, found 715.6046; $[M+Na+3H]^{4+}$ calcd for $C_{80}H_{135}N_{22}NaO_{41}P_2^{4+}$ 536.4635, found 536.9553) and ^{31}P NMR (202 MHz, H_2O, pH = 8.23) δ 20.10 (d, $^2J_{P\text{-}P}$ = 10.0 Hz, 1P), 13.44 (d, $^2J_{P\text{-}P}$ = 9.3 Hz, 1P).



Dizinc-bis(2-methylaminopicolylamino)-(tert-butyl)pyrenol hydrate (15)

The synthesis of compound **15** was reported previously.[13] All spectral data matched that which was previously reported.

IV. Assays for Chemical, Lysate, and Plasma Stability Studies of PCP-Peptides

Chemical Stability Studies

Buffer-Mediated Hydrolysis Conditions: PCP-peptides **PCP-5**, **PCP-7**, and **PCP-9** or their corresponding pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** (15.0 μL of a 1 mM stock solution in H_2O) and the desired buffer solution (50.0 μL of a 100 mM stock solution) were incubated at 37 ˚C for 24 hours in a 100 μL reaction volume, prior to HPLC analysis of a reaction aliquot (50.0 μL).

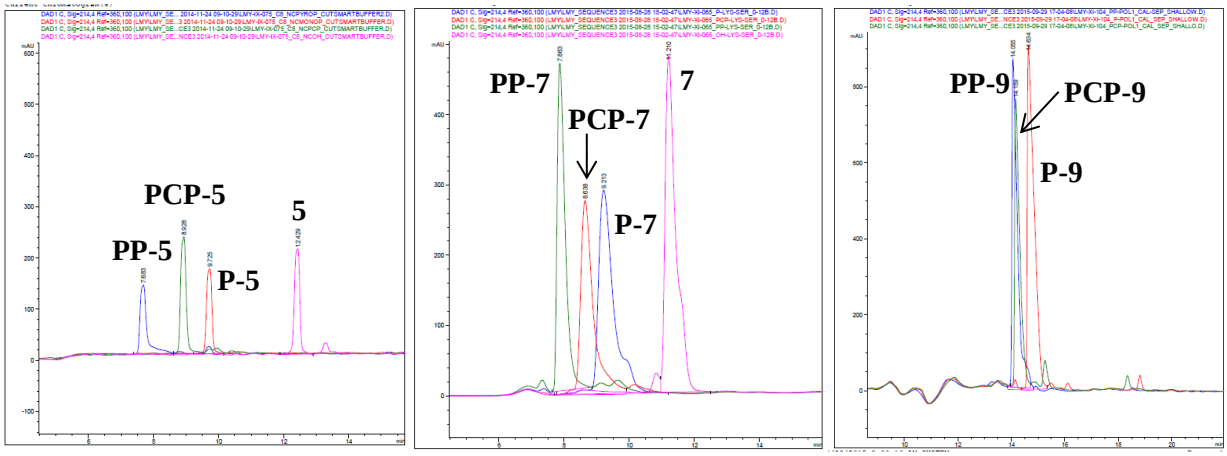
Base/Acid Hydrolysis Conditions: PCP-peptides **PCP-5**, **PCP-7**, and **PCP-9** or their corresponding pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** (15.0 μL of a 1 mM stock solution in H_2O) and the acidic or basic solution (85.0 μL of a 100 mM stock solution) were incubated at 37 ˚C for 24 hours in a 100 μL reaction volume, prior to HPLC analysis of a reaction aliquot (50.0 μL).

Metal-Mediated Hydrolysis Conditions: PCP-peptides **PCP-5**, **PCP-7**, and **PCP-9** or their corresponding pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** (10.0 μL of a 1 mM stock solution in H_2O), metal chloride salt (2.00 μL of a 10 mM stock solution) and a MOPS buffer solution (50.0 μL of a 100 mM stock solution, pH 7.9) were incubated at 37 ˚C in a 100 μL reaction volume. After incubating for 24 hours, the total reaction mixture was quenched with EDTA (0.250 μL of a 500 mM stock solution) prior to HPLC analysis of a reaction aliquot (50.0 μL).

Analytical HPLC was used to determine if any hydrolysis of the PCP-peptides to their corresponding fully dephosphorylated peptides occurred and to determine the percent hydrolysis of pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** to phosphopeptides **P-5**, **P-7**, and **P-9**. Any additional side products were also observed by analytical HPLC. The HPLC methods used for separation included the following (see traces below.):

- Method for Separation of **PP-5**, **PCP-5**, **P-5** & **5**: [C8; t = 0 min 10% of solvent B in solvent A; t = 20 min 30% of solvent B in solvent A; 1 mL/min; 280 nm; T_R(**PP-5**) = 7.683 min; T_R(**PCP-5**) = 8.928 min; T_R (**P-5**) = 9.725 min; T_R(**5**) = 12.429 min]
- Method for Separation of **PP-7**, **PCP-7**, **P-7**, & **7**: [C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 12% of solvent B in solvent A; 1 mL/min; 214 nm; T_R (**PP-7**) = 7.863 min; T_R (**PCP-7**) = 8.638 min; T_R (**P-7**) = 9.213 min; T_R (**7**) = 11.210 min]
- Method for Separation of **PP-9**. **PCP-9** & **P-9**: [C8; t = 0 min 3% of solvent B in solvent A; t = 25 min 10% of solvent B in solvent A; 1 mL/min; 140 nm; T_R(**PP-9**) = 14.055 min; T_R(**PCP-9**) = 14.159 min; T_R (**P-9**) = 14.634 min]

Percent hydrolysis was determined by the integrated area of any hydrolysis products at 214 or 280 nm divided by the total area of significant reaction components. Percent hydrolysis was normalized to time t = 0 (7.7% for **PP-5**, 4.9% for **PP-7**, and 0.0% for **PP-9**; No baseline hydrolysis for any PCP-peptide was observed.) for the buffer and base/acid condition or normalized to substrate in buffer alone for the metal cation assay.



Cell Lysate & Plasma Stability Studies:

Preparation of *S. cerevisiae* Lysates:

A wild-type (WT) strain with the genotype *his3Δ*1*leu2*Δ0*lys2Δ*0*ura3Δ*0 was used in this study. The WT strain was obtained from the Research Genetics Knockout Collection as the alpha mating type. Cells were grown at 30 ˚C to mid-log phase (OD_{600nm} = 0.550-0.650) in YPDA media. Cells were harvested by centrifugation at 3,000 rpm for 5 min at 4 ˚C. The cell pellets were rinsed with 50 mM Tris-HCl (pH 7.50; 10.0 mL) and isolated by centrifugation at 3,000 rpm for 5 min at 4 ˚C. The pellets were either used immediately for lysis or stored at – 80 ˚C until future use.

To prepare yeast lysates, one EDTA-free protease inhibitor cOmplete tablet (Roche Life Sciences) was added to a 1X Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.50; 10.0 mL) solution at 4 ˚C. All steps of the lysis were performed at 4 ˚C. The cell pellets were treated with the TBS solution (100 µL TBS solution per 10 OD's of cells), and mixed by vortexing. The cell suspension (300 µL) was transferred to screw-capped tubes, and glass beads were added. The cells were lysed by FastPrep (MP Biomedicals), using settings of 6.5 m/s for 3 x 60 seconds with 1 min rests between cycles. After the lysis was complete, a hole was poked in the bottom of the tubes, and the sample was removed from the glass beads by centrifugation at 6,000 rpm for 30 sec. Cellular debris was removed by centrifugation at 13,000 rpm for 20 min. The supernatant was removed and diluted with a 50% glycerol solution to a final glycerol concentration of 15%. Prior to diluting the lysate with glycerol, the protein concentration was determined by BCA kit (Pierce), with optimal assay conditions obtained with a protein concentration of 1.7-2.0 mg/mL. The lysates were stored at – 80 ˚C until future use.

Preparation of HeLa Lysates:

HeLa cells were obtained from ATTC (ATCC, CCL-2™ Cell Line). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (Gemini Bio-Products), L-glutamine (2 mM; Life Technologies), and antibiotics [penicillin (100 units/mL) and streptomycin (100 µg/mL); Life Technologies] at 37 ˚C and 5% CO_2.[5] Plates (10 cm) were aspirated and cells were washed with a 1X phosphate-buffered saline (PBS; 2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 138 mM sodium chloride, 8.06 mM sodium

phosphate dibasic, pH 7.0-7.2; Life Technologies; 5 mL), trypsinized (1 mL of 0.250% solution; Life Technologies) and split in a 1:4 ratio.

To prepare mammalian lysates, one EDTA-free protease inhibitor cOmplete tablet (Roche Life Sciences) was added to a 1X TBS (10 mL) solution at 4 ˚C. All steps of the lysis were performed at 4 ˚C. The TBS solution (1 mL) containing inhibitors was added to plates with cells at a concentration of $5.9x10^6$ cells per plate. After incubating the plate for 10 min in TBS, the cells were removed from the plate with a cell scraper, and the solution was transferred to a 5 mL Eppendorff tube. The cells were vortexed (10 x 5 sec) with resting on ice between cycles. Cellular debris was removed by centrifugation at 13,000 rpm for 10 min at 4 ˚C. The supernatant was removed and diluted with a 50% glycerol solution to a final glycerol concentration of 15%. The protein concentration was determined by BCA kit (Pierce), with optimal assay conditions obtained with a protein concentration of 0.7-0.8 mg/mL. The lysates were stored at – 80 ˚C until future use.

Protocol for Peptide Dephosphorylation Assay in Cell Lysates:

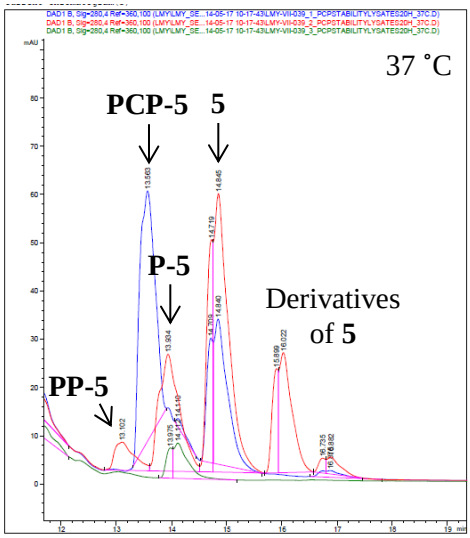
The cell lysate (For *S. cerevisiae,* 64.0 μg, 50.0 μL of 1.27 mg/mL stock stored in 15% glycerol; For HeLa, 40.0 μg, 50.0 μL of 0.80 mg/mL stock stored in 15% glycerol), either **PCP-5** or **PP-5** (17.0 μL of a 0.710 or 0.750 mM stock solution, stock solutions calibrated by analytical HPLC using 214 nm absorbance[14]), and Tris-HCl buffer (pH 7.50; 7.50 μL of a 1.00 M stock solution) were incubated at 37 ˚C for 1 to 24 hours in a 150 μL reaction volume. For the samples containing the heat denatured lysate, the yeast lysate was boiled for 5 minutes at 100 ˚C prior to incubation with peptide for 4 or 24 hours. For samples treated with phosphatase inhibitors, the buffered lysate solution was pre-treated with both EDTA (3.00 μL of a 100 mM stock solution) in H_2O and 5% phosphatase inhibitor cocktail 2 (Sigma Aldrich) in H_2O prior to addition of the substrate. After incubating the samples for the desired time, the samples were quenched by boiling for 5 minutes, followed by centrifugation at 13,000 rpm for 0.5 min. The samples were then passed through a 0.22 μm syringe filter to remove the denatured enzymes. An aliquot (60.0 μL) of the reaction mixture was analyzed by analytical HPLC (see method below) for hydrolysis of the starting material. The integrated area of the product peaks (dephosphorylated and derivative products) at 280 nm was divided by the total area of all significant peptide components in the reaction mixture to determine the percent hydrolysis. Percent hydrolysis was normalized to starting material incubated in buffer under standard reaction conditions for the same time point taken.

Protocol for Peptide Dephosphorylation Assay in Human Plasma:

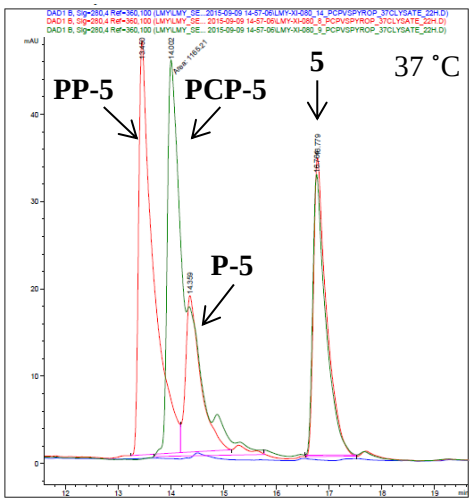
Human plasma was obtained from Sigma Aldrich as a lyophilized powder from a 5 mL original volume. The powder was resuspended in 5 mL of water, and 125 μL of the plasma serum was added to a solution containing peptide **PCP-5** (208 μL of a 1.20 mM stock calibrated by HPLC) and Tris-HCl buffer (pH 7.50, 25 μL of a 1.00 M stock stolution) in a 500 μL reaction volume. The sample was transferred to an NMR tube and heated at 37 ˚C for 4 to 48 hours. After 4, 24, and 48 hours of incubation, the sample was analyzed by ^{31}P NMR (202 MHz, 512 scans) for hydrolysis of the methylene bisphosphonate moiety. ^{31}P spectra were referenced to a tetramethylphosphonium bromide (Me_4PBr) standard (22.77 ppm) in a D_2O capillary insert, which was used to lock the samples that were in non-deuterated water.

Analytical HPLC Assays for Separation of Peptides for Cell Lysate Studies:

PCP-5 and **PP-5** Starting Materials: *Yeast Lysate*: C18; t = 0 min 0% of solvent B in solvent A; t = 20 min 47% of solvent B in solvent A; 1 mL/min; 280 nm; T_R (**PP-5**) = 13.825 min, T_R (**PCP-5**) = 14.737 min, T_R (**P-5**) = 15.727 min, T_R (**5**) = 15.727 min, and T_R (Derivatives of **5**) = 17.182 min & 18.084 min. *HeLa Lysate*: C18; t = 0 min 0% of solvent B in solvent A; t = 5 min 0% of solvent B in solvent A; t = 20 min 30% of solvent B in solvent A; 1 mL/min; 280 nm; T_R (**PP-5**) = 13.450 min, T_R (**PCP-5**) = 14.002 min, T_R (**P-5**) = 14.359 min, and T_R (**5**) = 16.779 min.



Blue = **PCP-5** + Yeast Lysate
Red = **PP-5** + Yeast Lysate
Green = Yeast Lysate Small
Molecule Background



Blue = **PCP-5** + HeLa Lysate
Red = **PP-5** + HeLa Lysate
Blue = HeLa Lysate Small
Molecule Background

V. Protocols for Protein Ligations: Maleimide and Glutaraldehyde Conjugations

Protocol for Maleimide Conjugation of Peptides **PCP-8** and **8** to M.A.-BSA:

The maleimide conjugation was performed following a modified procedure from the technical bulletin.[15] Maleimide-activated bovine serium albumin (M.A.-BSA, Imject BSA) was purchased from Sigma Aldrich and used as received. 50.0 μL of M.A.-BSA stock (50.0 μg protein, 17 mol maleimide/mol of BSA stock, 125 nmol maleimide) was added to an eppendorf tube followed by 107-117 μL of 2x ligation buffer (2x concentrations = 200 mM sodium phosphate, 300 mM NaCl, 200 mM ethylenediaminetetraacetic acid, pH 7.30) and lastly by peptide **PCP-8** (57.0 μL of 4.62 mM stock calibrated by analytical HPLC,[13] 263 nmol, 2.1 equivalents) or **8** (67.0 μL of 4.62 mM stock calibrated by analytical HPLC,[13] 263 nmol, 2.1 equivalents). After rotating the samples for 2 hours at room temperature, samples were removed and placed in a 0.1-0.5 mL volume 7 kDa Slide-A-Lyzer™ dialysis cassette (ThermoFisher Scientific) to dialyze overnight in 23 mM NaCl and 20 mM Tris (pH 7.50) buffer (2.00 L) volume. The product was precipitated with cold acetone (6:1 equivalents acetone:reaction mixture) for 1 hour at -20 C˚ and centrifuged at 4,500 rpm for 15 minutes at 4 ˚C to isolate protein conjugates BSA-**PCP-8** and BSA-**8**. The samples were allowed to air dry to remove any residual acetone. Due to the variable number of peptide chains that could be added to the protein during ligation affecting any protein concentration assay read-out, the isolated protein from this ligation was assumed to be 50.0 μg based on the initial starting material amount. After the samples were fully dry, the protein (~50.0 μg) was resuspended in water (100 μL) with 6x loading dye (20.0 μL) and stored at -20 ˚C prior to analysis by gel electrophoresis.

Protocol for Glutaraldehyde Conjugation of Peptides **PCP-7**, **PP-7**, and **7** to GST:

The glutaraldehyde ligation was performed following a modified procedure from Hermanson, G.[16] Glutathione S-transferases (GST) was expressed in and isolated from *E. Coli* following standard procedures.[13]

To express and isolate GST, *E. Coli* (BL 21), which contained a GEX-2T plasmid, were grown at 37 ˚C to mid-log phase (OD_{600nm} ~0.500) in LB media (200 mL) with ampicillin (100 μg/mL). Once the cells were in log-phase, production of GST was induced with IPTG (100 μM final concentration) and incubated for 3 hours at 30 ˚C. Cells were harvested by centrifugation at 4,500 rpm for 10 min at 4 ˚C. The cell pellets were rinsed with 50 mM Tris-HCl (pH 7.50; 10.0 mL) and isolated by centrifugation at 4,500 rpm for 5 min at 4 ˚C. The cell pellet was resuspended in 2 mL of lysis buffer containing 50 mM Tris HCl (pH 7.50), 150 mM NaCl, 0.05% Triton-X, Roche cOmplete protease inhibitor, and lysozyme (20.0 uL of a 50.0 μg/mL stock, pH 8.00 buffer), and the cells were rested on ice for 45 minutes in lysis buffer. Samples were then sonicated (10 x 5 sec sonicate, then 2 minutes rest) and centrifuged at 13,200 rpm for 20 minutes at 4 ˚C to pellet cellular debris. The supernatant was removed and added to 100 μL of glutathione sepharose beads per 1 mL of lysate. The samples were incubated with shaking for 2 hours at 4 ˚C. The samples were then spun at 6000 rpm for 2 minutes at 4 ˚C to precipitate beads. The supernatant was removed and discarded. The beads were then washed with lysis buffer (1.5 mL/100 μL beads, 1 x 10 minutes incubation with shaking, 4 ˚C) and then spun at 6000 rpm for 2 minutes at 4 ˚C to precipitate beads. The supernatant was removed and discarded. The beads were then washed with elution buffer without glutathione (50 mM Tris, pH 8.00, 1.25 mL/100 μL beads, 1 x 10 minutes incubation with

shaking, 4 ˚C) and then spun at 6000 rpm for 2 minutes at 4 ˚C to precipitate beads. The supernatant was removed and discarded. The beads were then treated with elution buffer containing glutathione (50 mM Tris, 10 mM reduced glutathione, pH 8.00, 1 mL/1 mL of beads) and eluted for 4 hours at 4 ˚C. The samples were then centrifuged at 2000 rpm for 5 minutes, and the supernatant containing the isolated GST was saved. The concentration of GST in the sample was determined by BCA kit (Pierce).

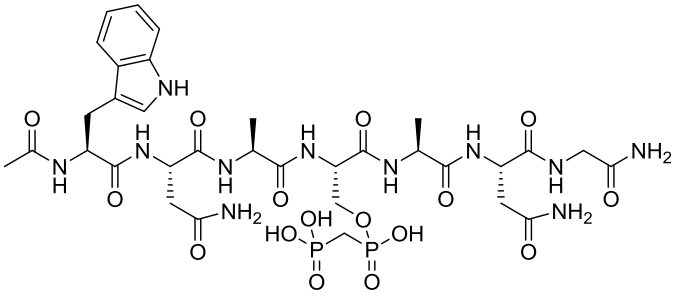
To perform the GST ligation, 50.0 μL of GST protein stock (4.50 nmol, 117 μg as determined by BCA kit) and 25.0 μL of peptide **PCP-7**, **PP-7**, or **7** stocks (~10 mM solutions, calibrated by analytical HPLC[13]) were added to eppendorf tubes at 4 ˚C. After addition of the substrates, 50.0 μL of carbonate buffer (Initial Stock Solution = 200 mM Na_2CO_3, 300 mM NaCl, pH adjusted to 8.50) was added to the samples, followed lastly by 4.00 μL of 25% glutaraldehyde solution (Sigma Aldrich, used as received). For the sample containing the GST-GST dimer control, 25.0 μL of water was added instead of peptide solution with 2.00 μL of 25% glutaraldehyde solution added. All samples were incubated with shaking overnight at 4 ˚C. After overnight incubation to reduce the resulting imines, 1.00 mg of $NaCNBH_3$ was added to each tube at 4 ˚C. After reducing the sample for 1 hour, samples were removed and placed in a 0.1-0.5 mL volume 7 kDa Slide-A-Lyzer™ dialysis cassette (ThermoFisher Scientific) to dialyze overnight in 23 mM NaCl and 20 mM Tris (pH 7.50) buffer (2.00 L) volume. The product was precipitated with cold acetone (6:1 equivalents acetone:reaction mixture) for 3 hours at 20 ˚C and centrifuged at 4,500 rpm for 15 minutes at 4 ˚C to isolate protein conjugates GST-GST, GST-**PCP-7**, GST-**PP-7,** and GST-**7**. The samples were allowed to air dry to remove any residual acetone. Due to the variable number of peptide chains that could be added to the protein during ligation affecting the protein concentration assay read-out, the isolated protein from this ligation was assumed to be 117 μg based on the initial starting material amount. After the samples were fully dry, the protein (~117 μg) was resuspended in water (25.0 μL) with 6x loading dye (8.40 μL) and 20x DTT (1.75 μL) and analyzed by gel electrophoresis.

VI. Protocol for Gel Staining with 15 to Visualize PCP-Peptide-Protein Conjugates

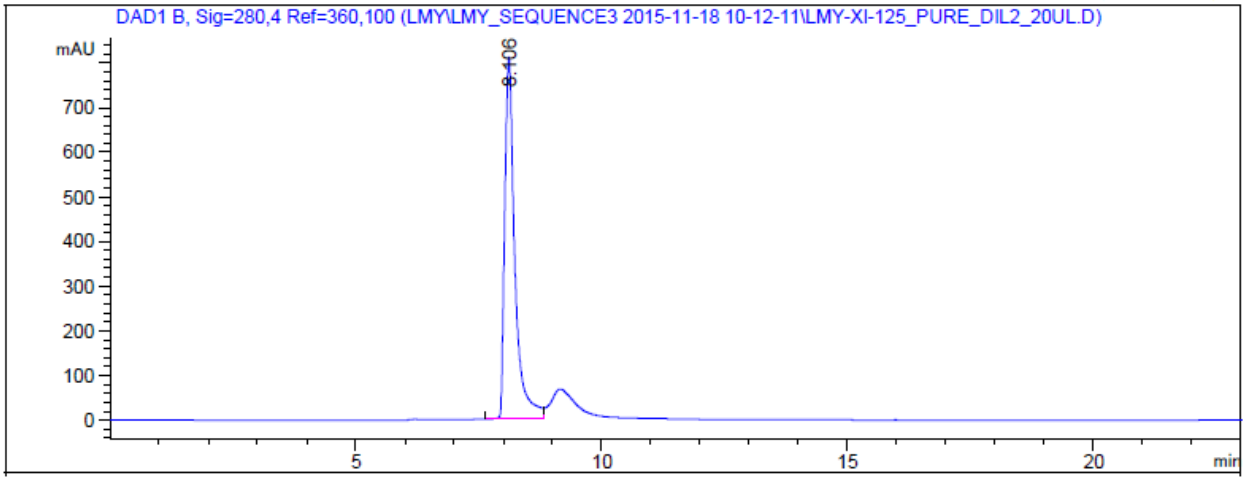
The protocol for gel staining with **15** was conducted according to Williams, *et. al.*[13] Briefly, after gel electrophoresis (Criterion Bis-Tris Mini-Gel 10%, XT-MES buffer, 150 V, 1.5 hours), the acrylamide gel was fixed with 50% MeOH, 10% acetic acid in water (100 mL for 30 minutes, then 100 mL overnight). After washing with water (3 x 10mL x 10min), the gel was treated with DMSO solution [200 μM sodium phosphate, 200 μM $ZnBr_2$ in 19:1 DMSO:HEPES (100 mM, pH = 7.16), 2 x 50 mL x 30 min]. The gel was then treated with staining solution [6.5 μM **15** in 1:1 DMSO:HEPES (100 mM, pH = 7.16), 40 mL, 1h]. After treating with the staining solution, the gel was treated with fresh DMSO solution (2 x 50 mL x 30 min). The gel was then re-exposed to the original staining solution previously used (1h) then treated with fresh DMSO solution (50 mL, 30 min then 50 mL, 30 min to overnight, as needed. Typically 4-8 hours destaining of the gel resulted in optimal protein signal over background dye remaining in the gel.). The gel was then transilluminated at 365 nm and visualized with a Protein Simple blue filter (460 nm ± 40 nm) for the camera.

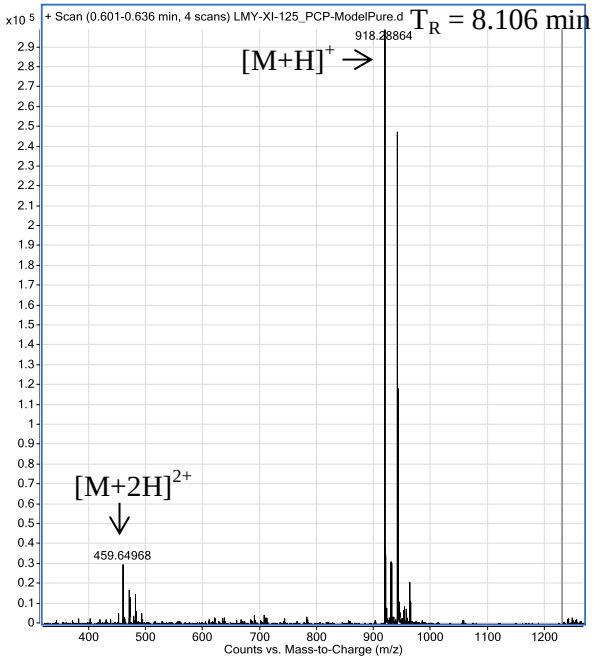
VII. Peptide HPLC Traces and Mass Spectrometry Characterization

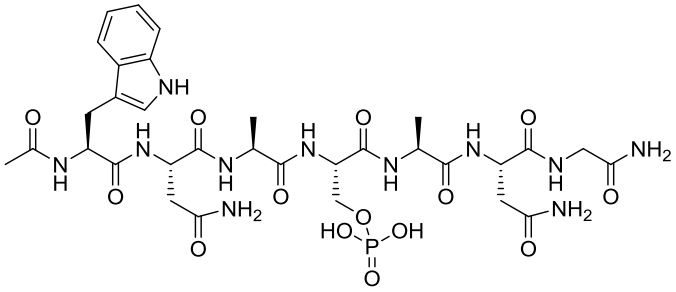
Unless otherwise noted, analytical HPLC traces shown are of HPLC purified material.



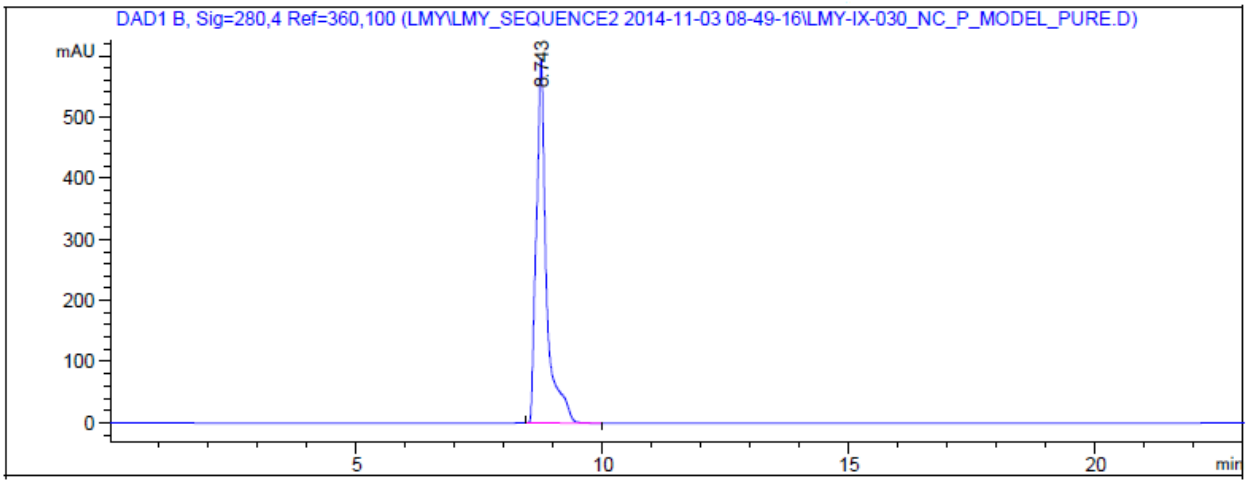
Ac-Trp-Asn-Ala-[PcPSer]-Ala-Asn-Gly-CONH$_2$ (PCP-5)

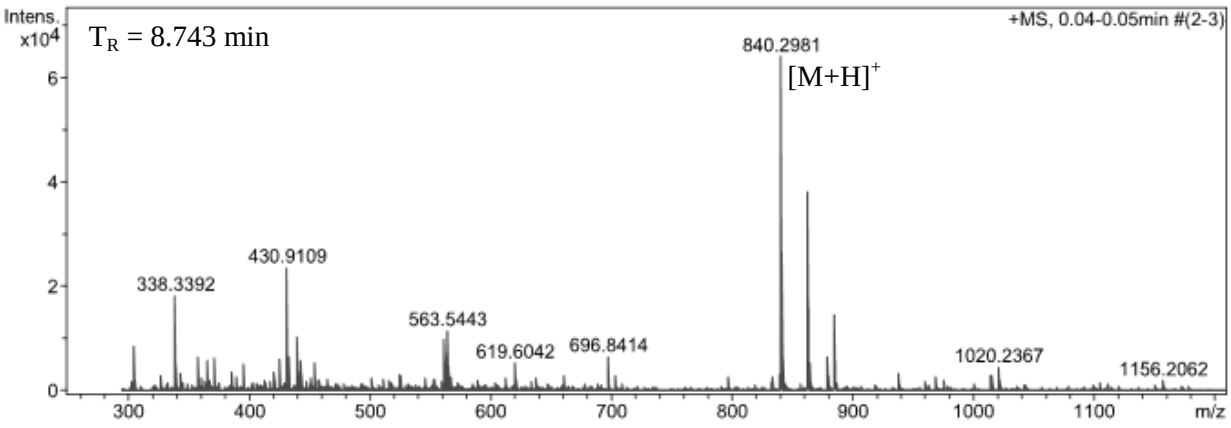


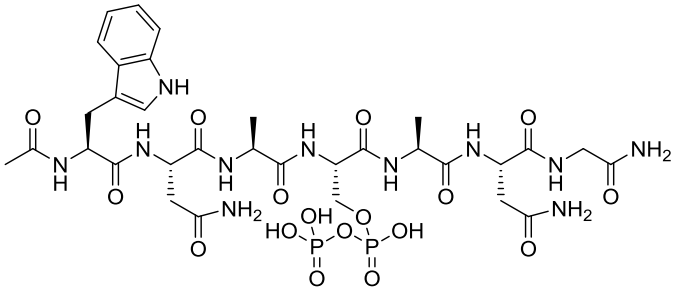




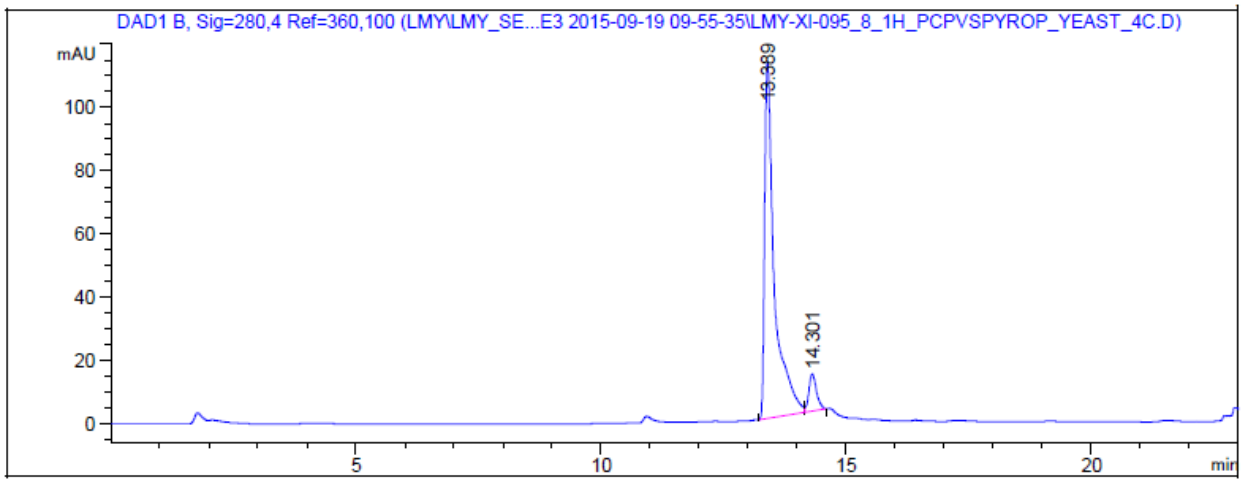
Ac-Trp-Asn-Ala-[PSer]-Ala-Asn-Gly-CONH$_2$ (P-5)

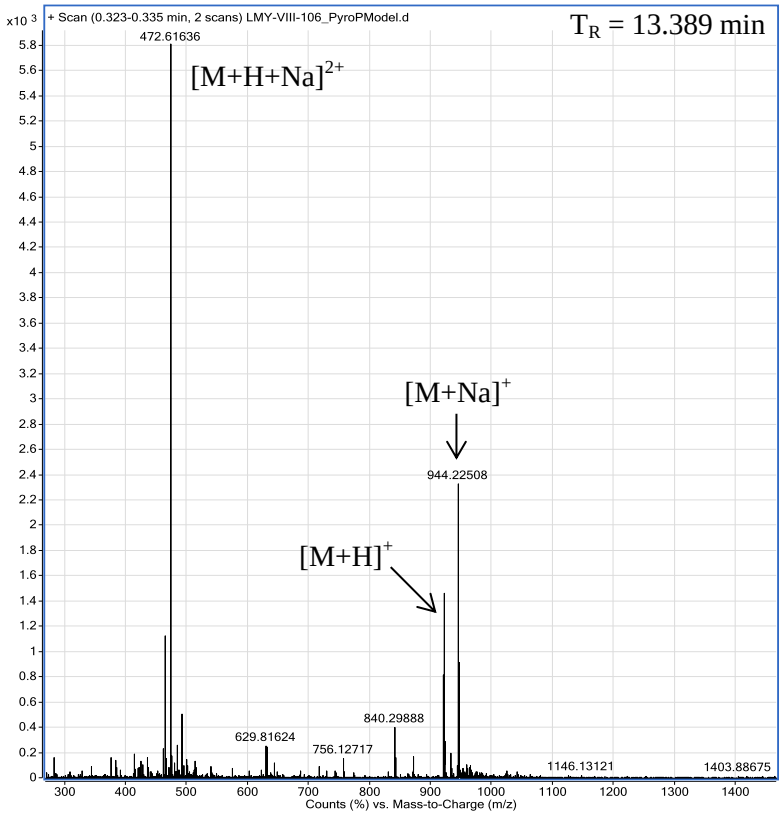


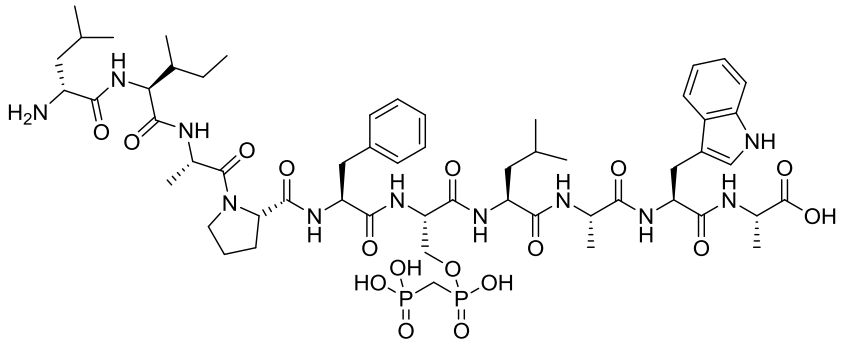




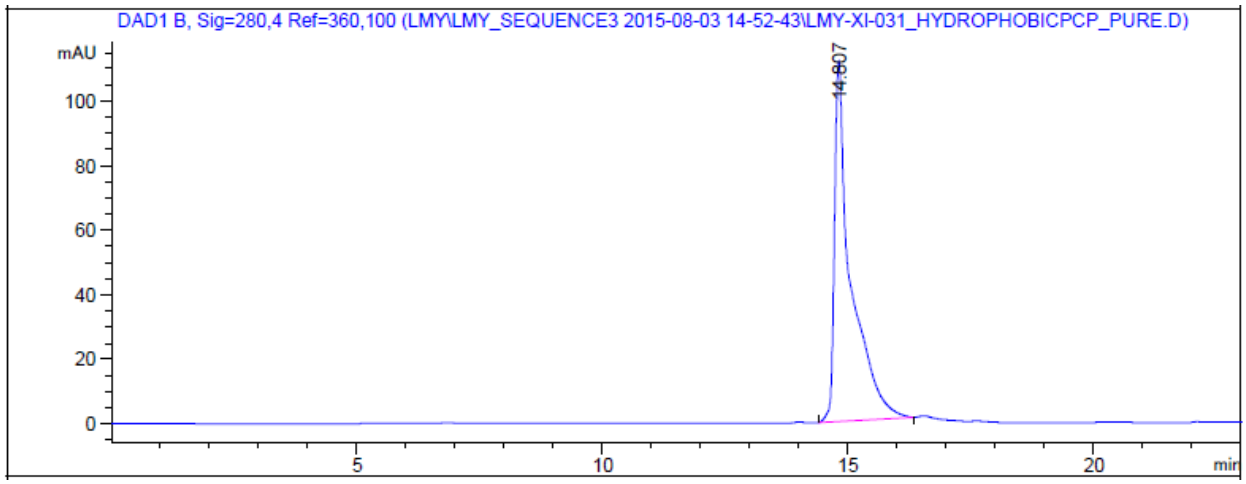
Ac-Trp-Asn-Ala-[PyroP-Ser]-Ala-Asn-Gly-CONH$_2$ (PP-5)

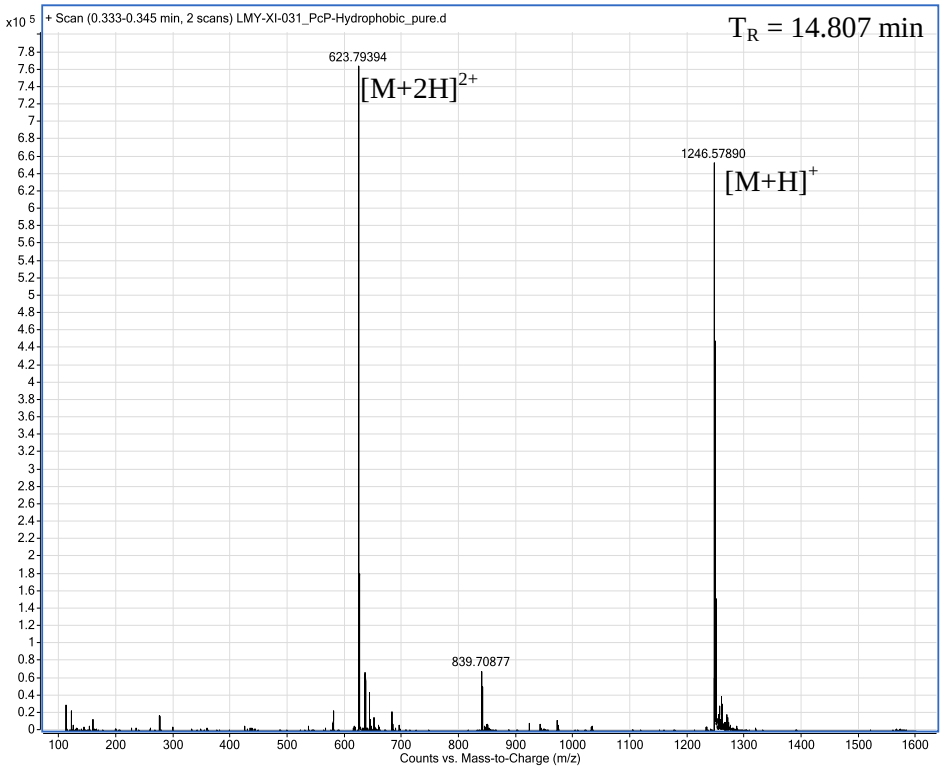


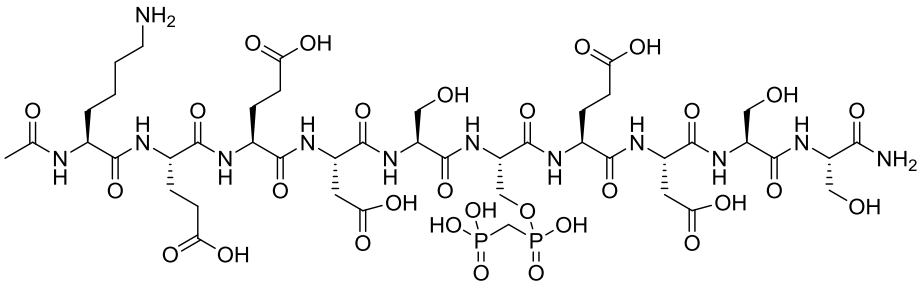




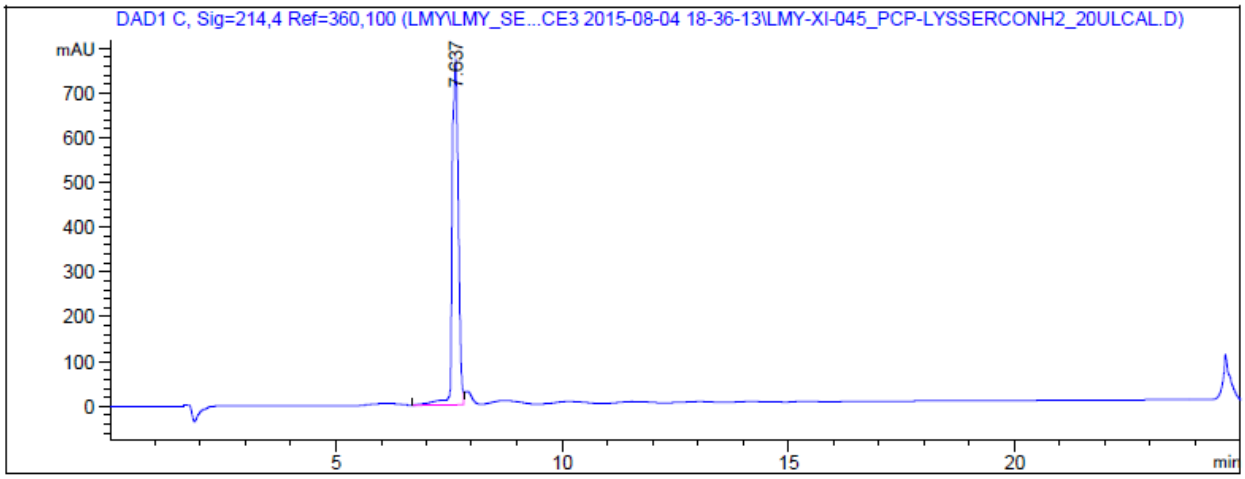
H_2N-Leu-Ile-Ala-Pro-Phe-[PcPSer]-Leu-Ala-Trp-Ala-COOH (PCP-6)

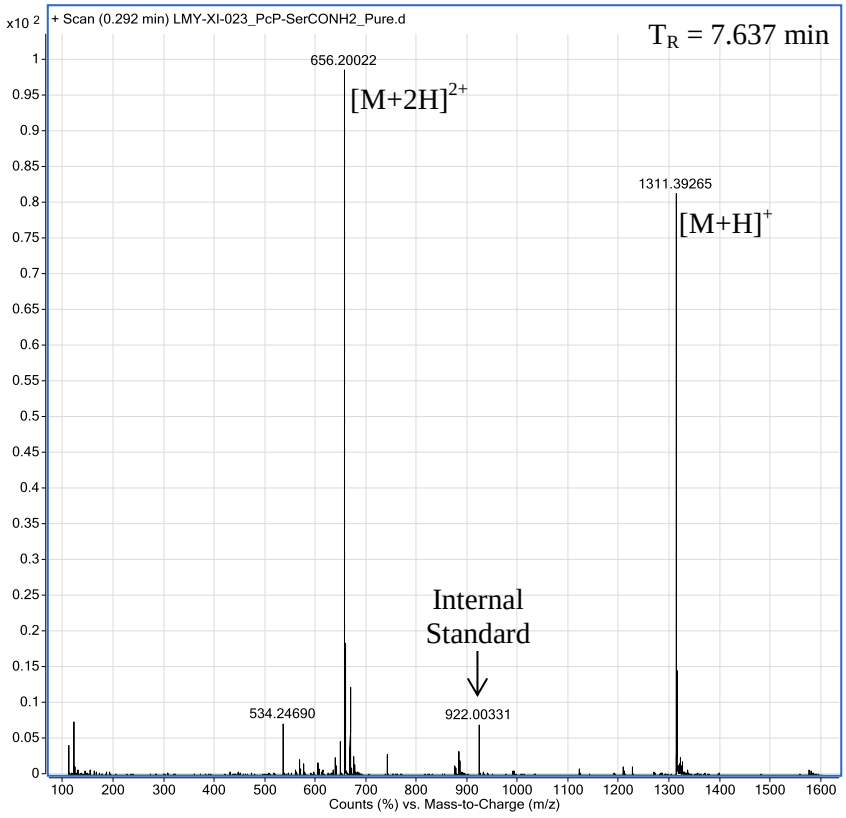


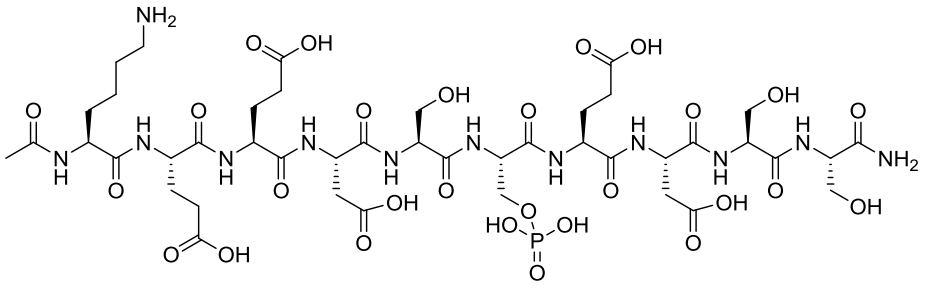




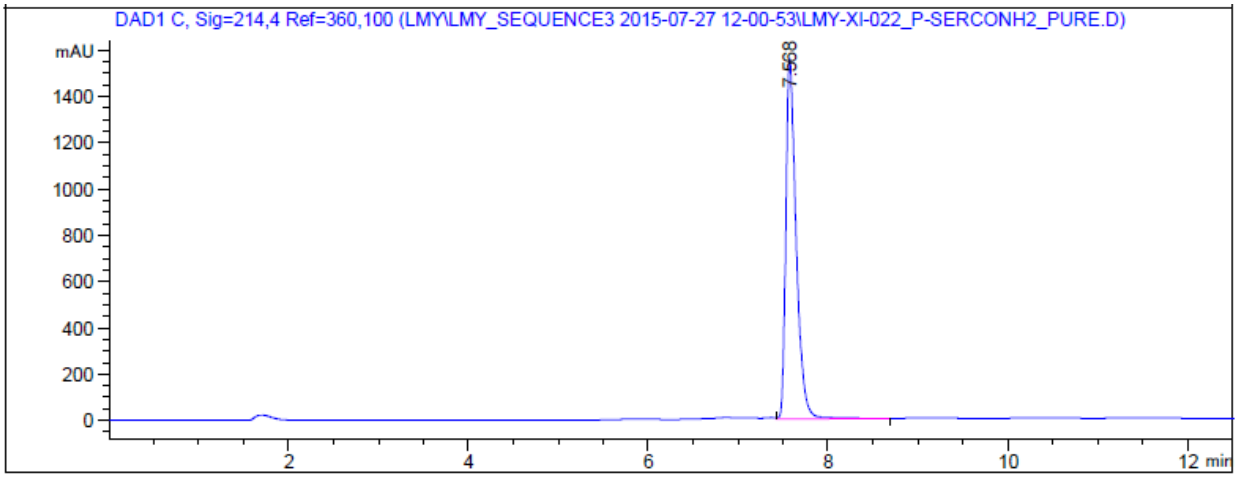
Ac-Lys-Glu-Glu-Asp-Ser-[PcPSer]-Glu-Asp-Ser-Ser-CONH$_2$ (PCP-7)

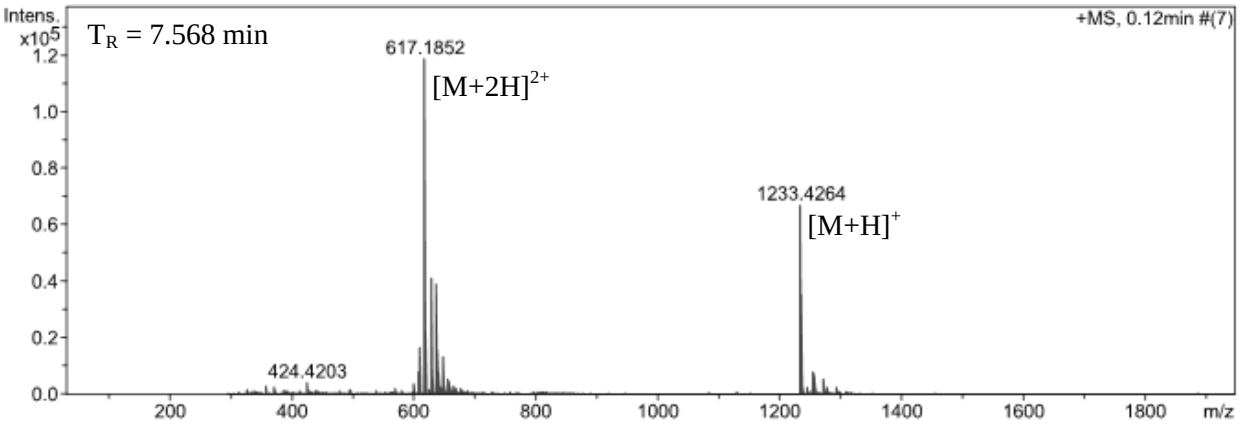


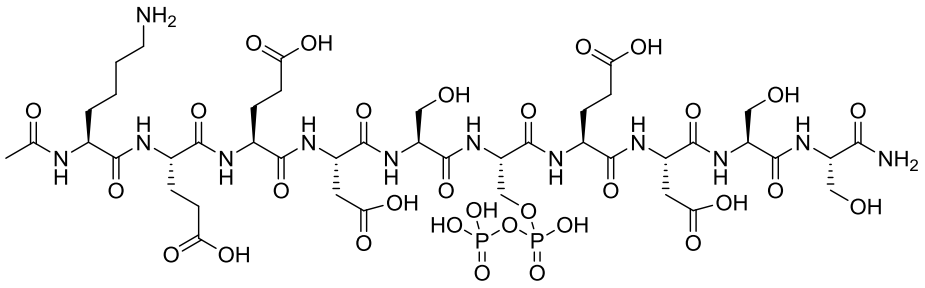




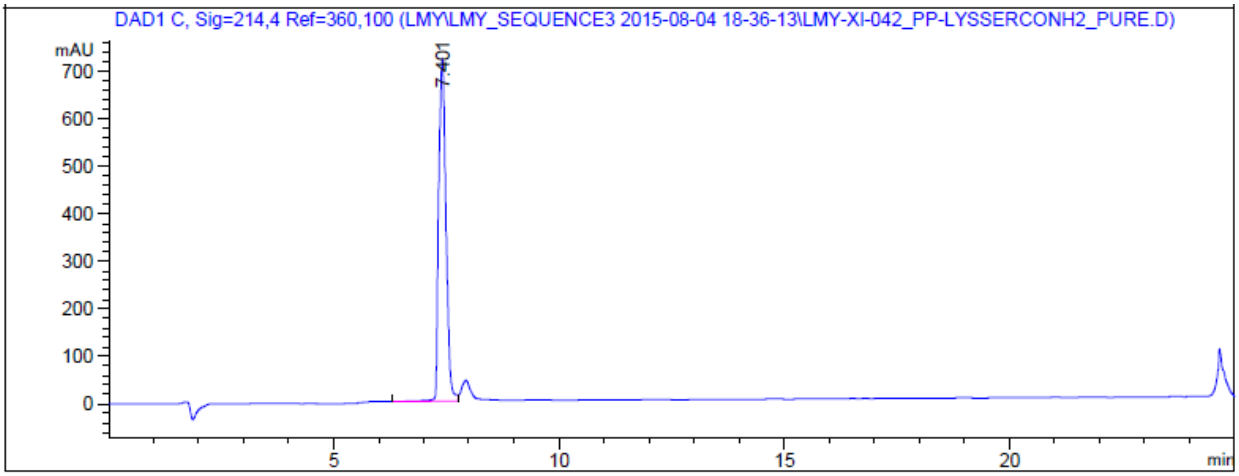
Ac-Lys-Glu-Glu-Asp-Ser-[PSer]-Glu-Asp-Ser-Ser-CONH$_2$ (P-7)

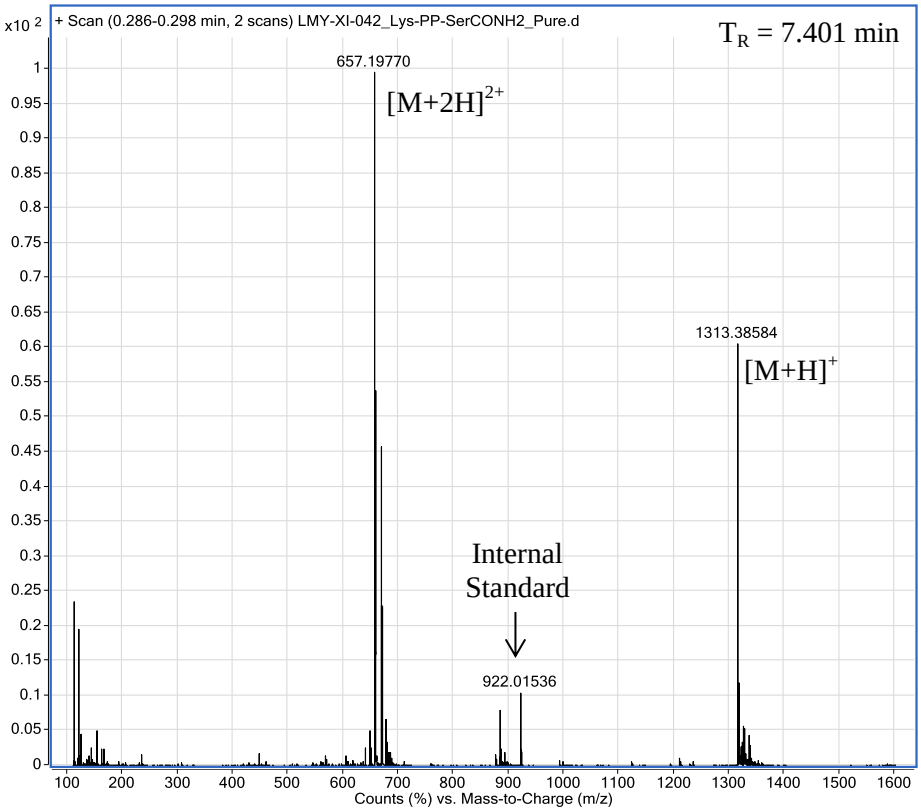


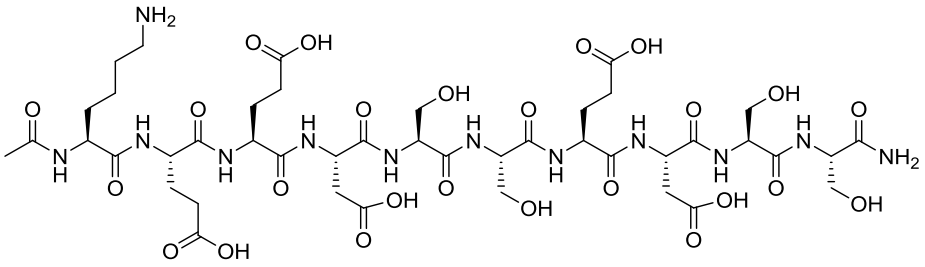




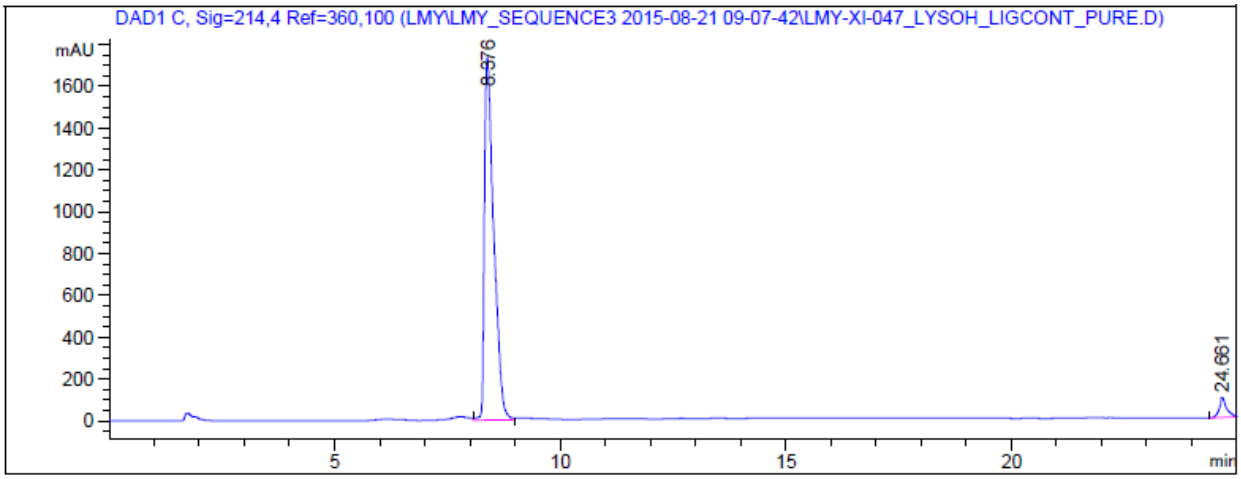
Ac-Lys-Glu-Glu-Asp-Ser-[PyroPSer]-Glu-Asp-Ser-Ser-CONH$_2$ (PP-7)

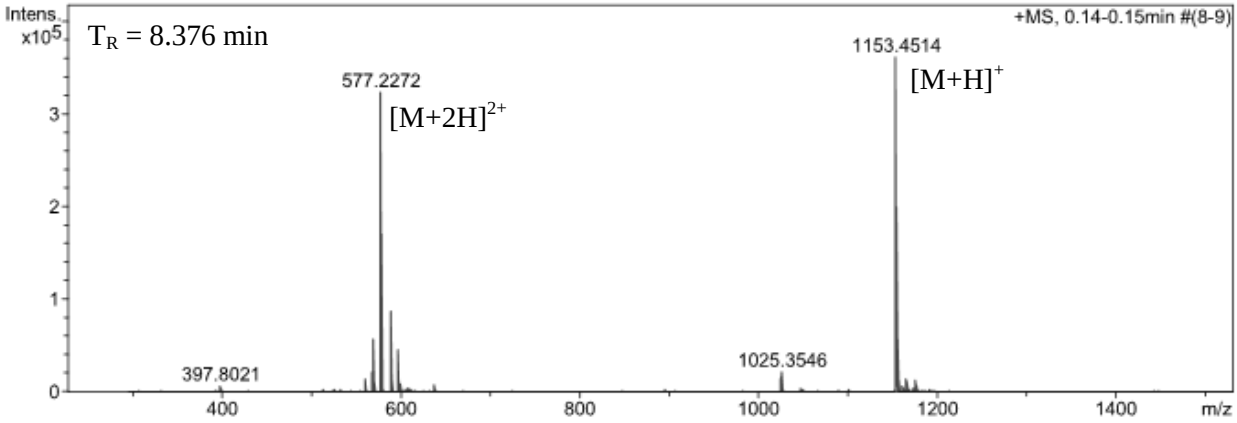


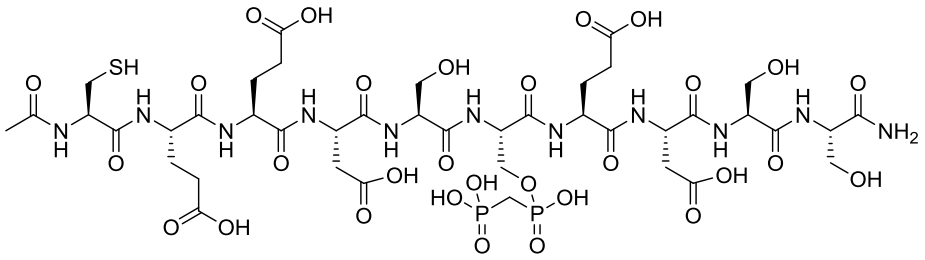




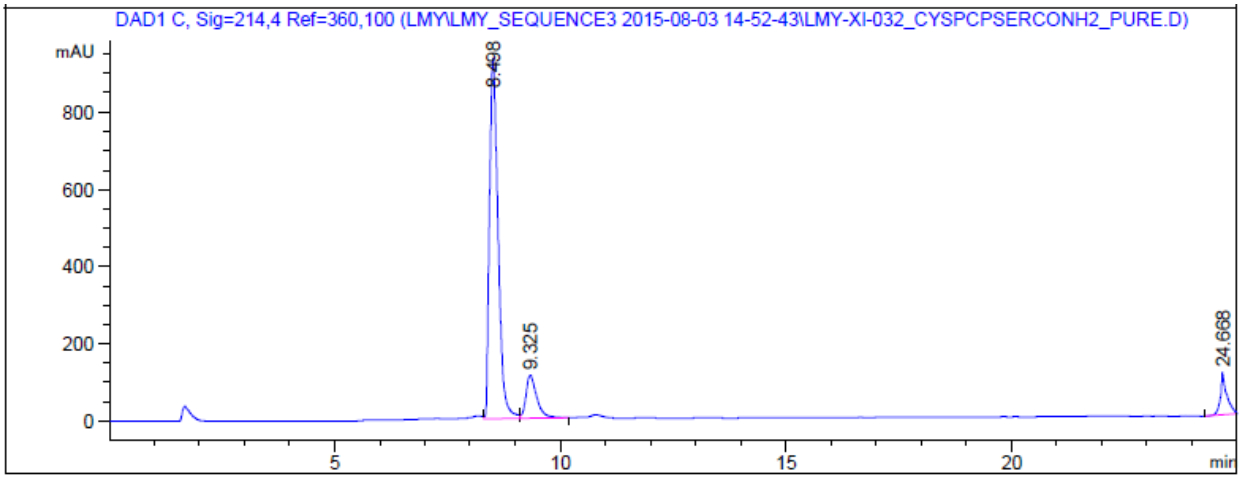
Ac-Lys-Glu-Glu-Asp-Ser-Ser-Glu-Asp-Ser-Ser-CONH$_2$ (7)

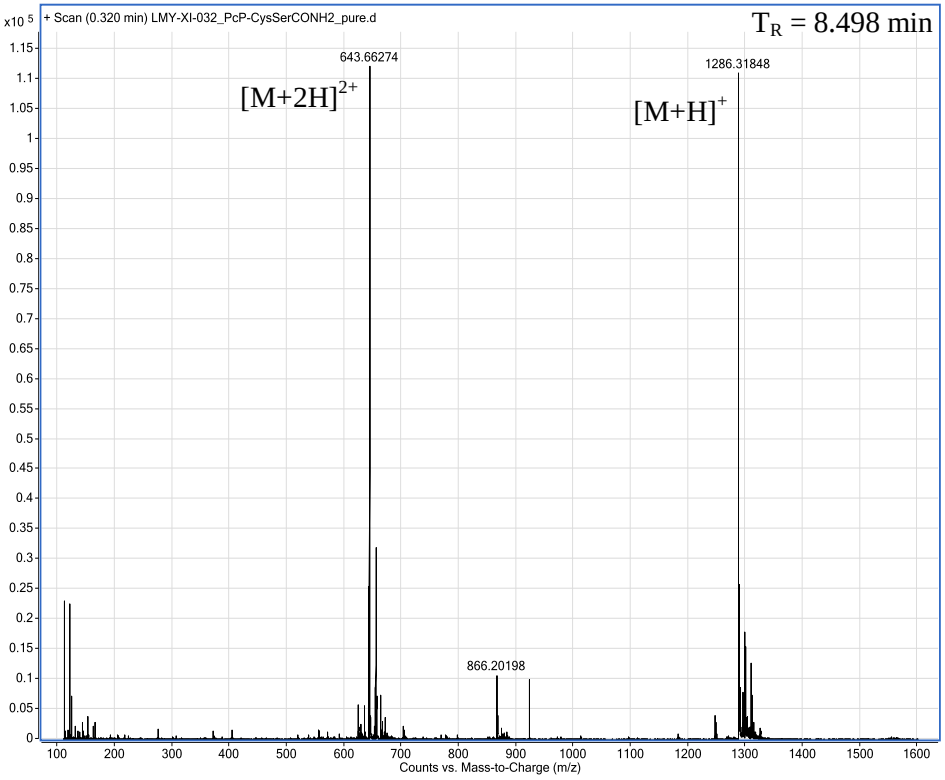


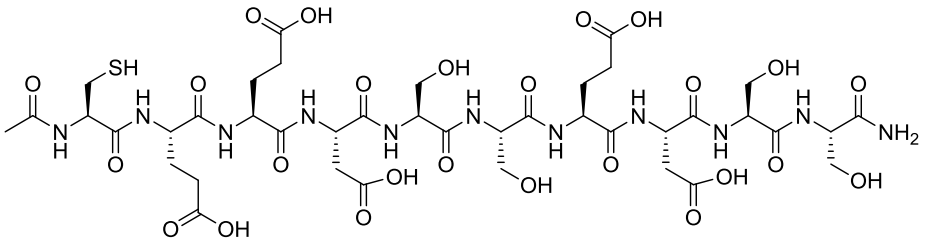




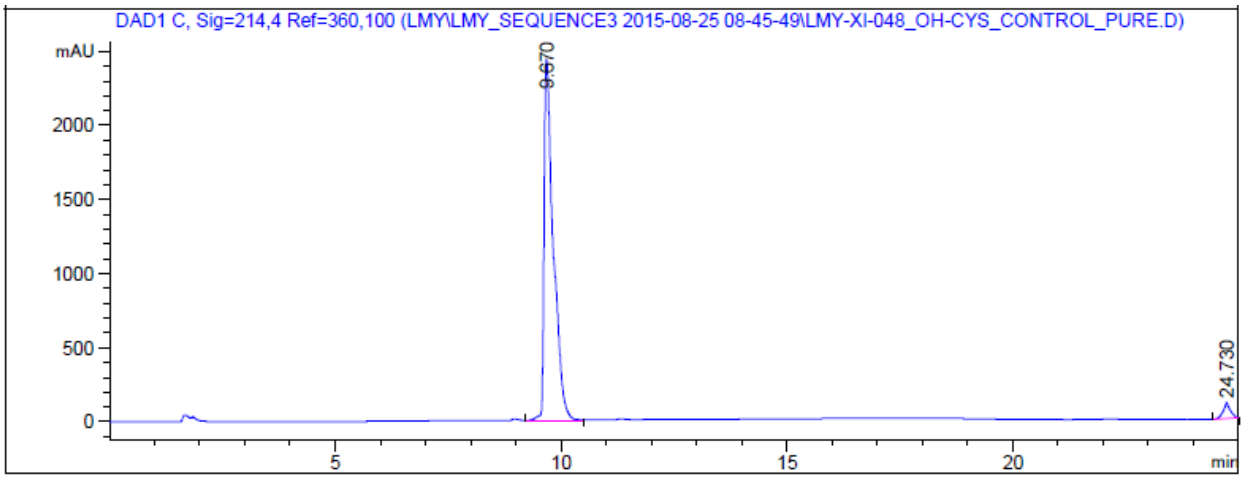
Ac-Cys-Glu-Glu-Asp-Ser-[PcPSer]-Glu-Asp-Ser-Ser-CONH$_2$ (PCP-8)

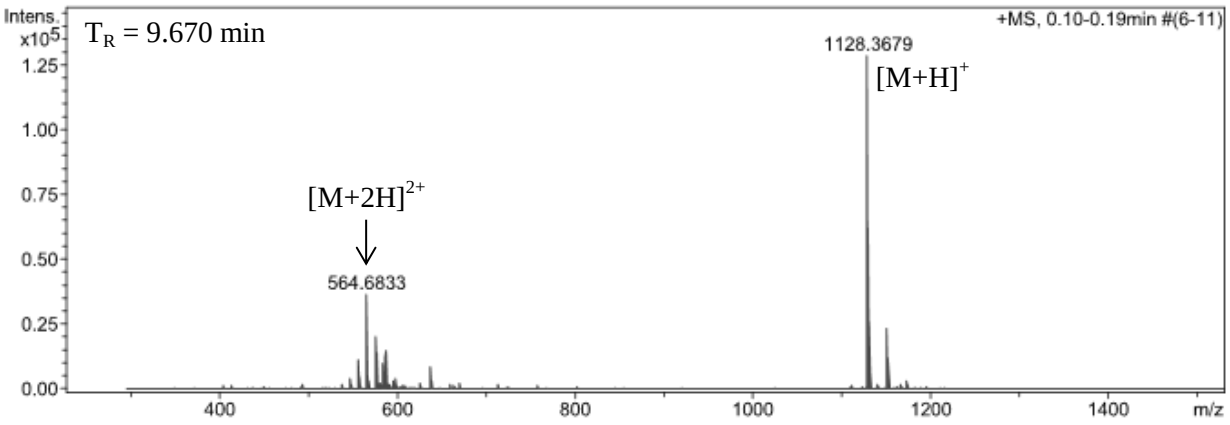


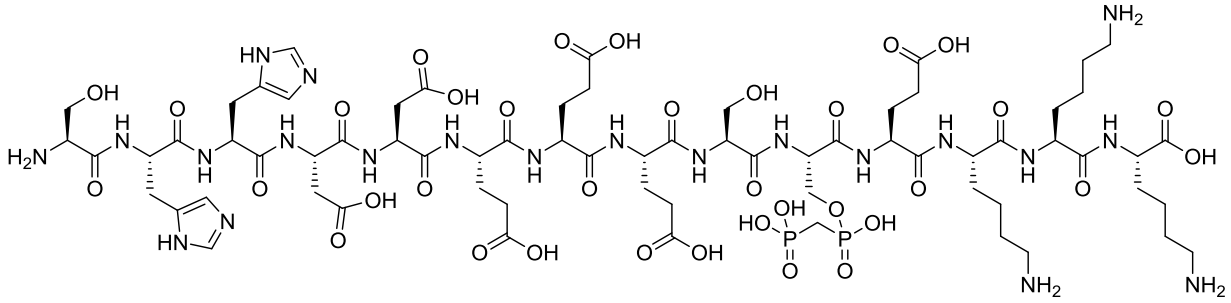




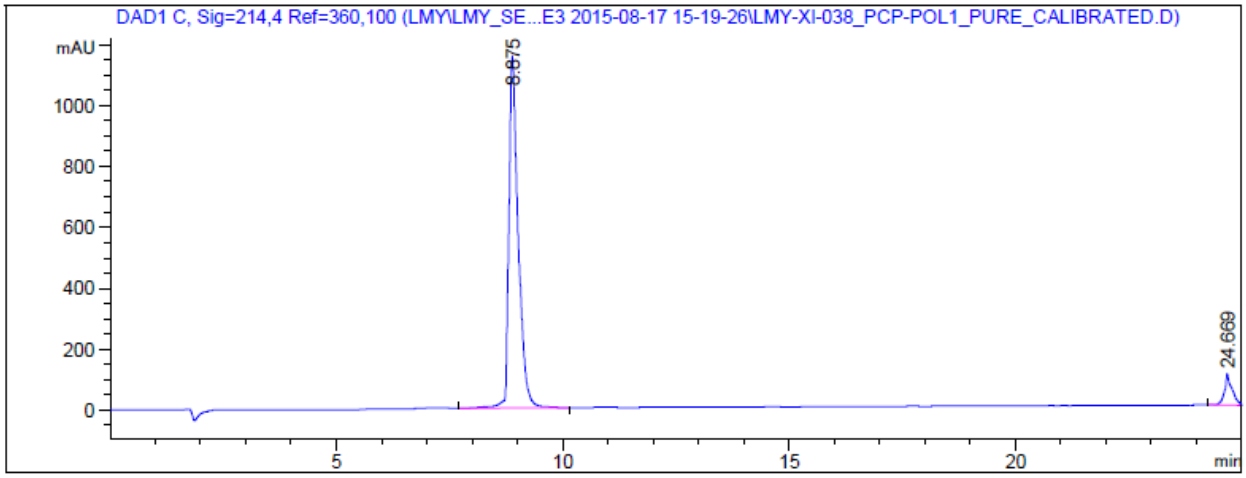
Ac-Cys-Glu-Glu-Asp-Ser-Ser-Glu-Asp-Ser-Ser-CONH$_2$ (8)

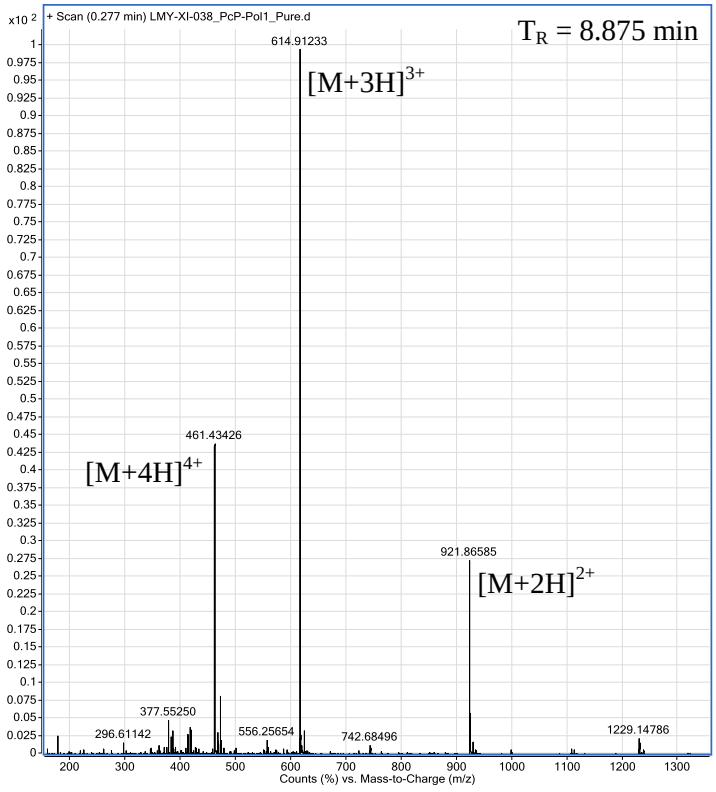


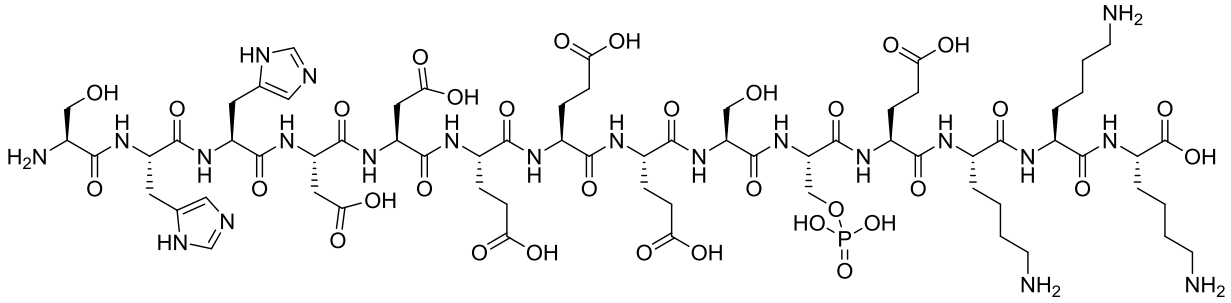




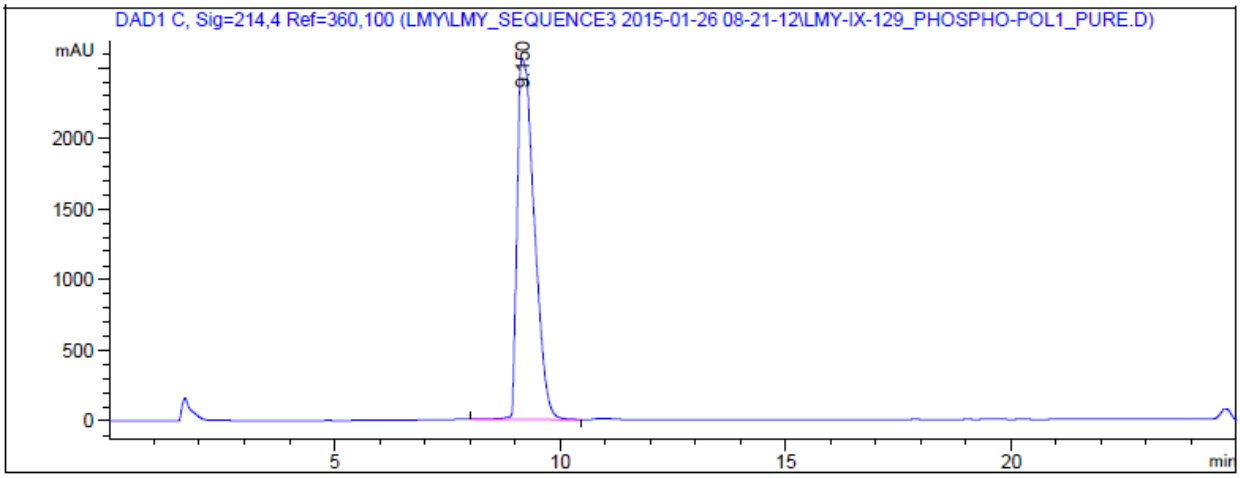
Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PcPSer]-Glu-Lys-Lys-Lys (PCP-9)

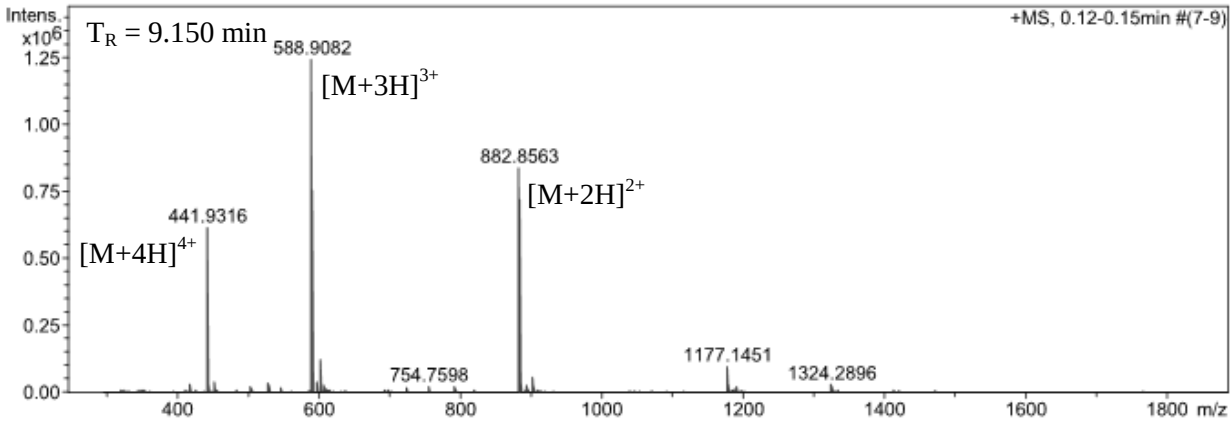


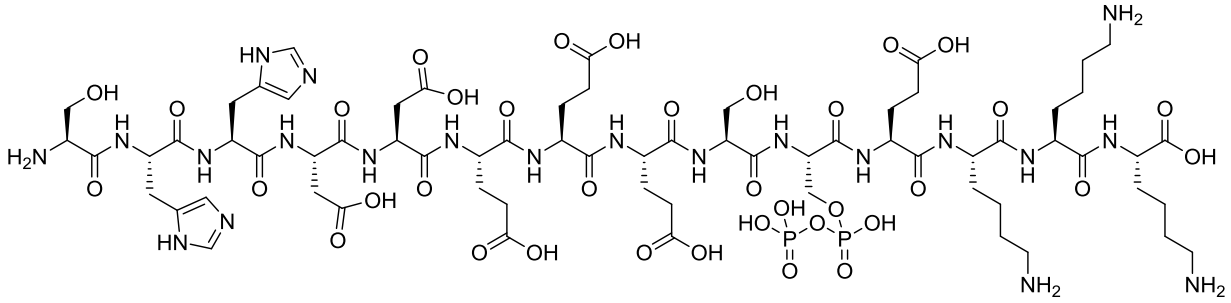




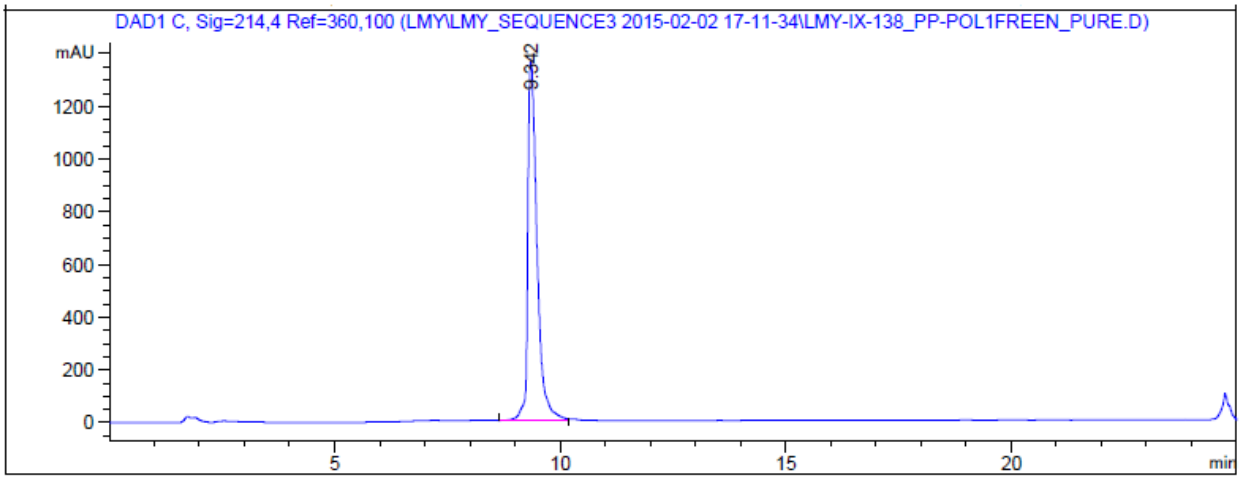
Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PSer]-Glu-Lys-Lys-Lys (P-9)

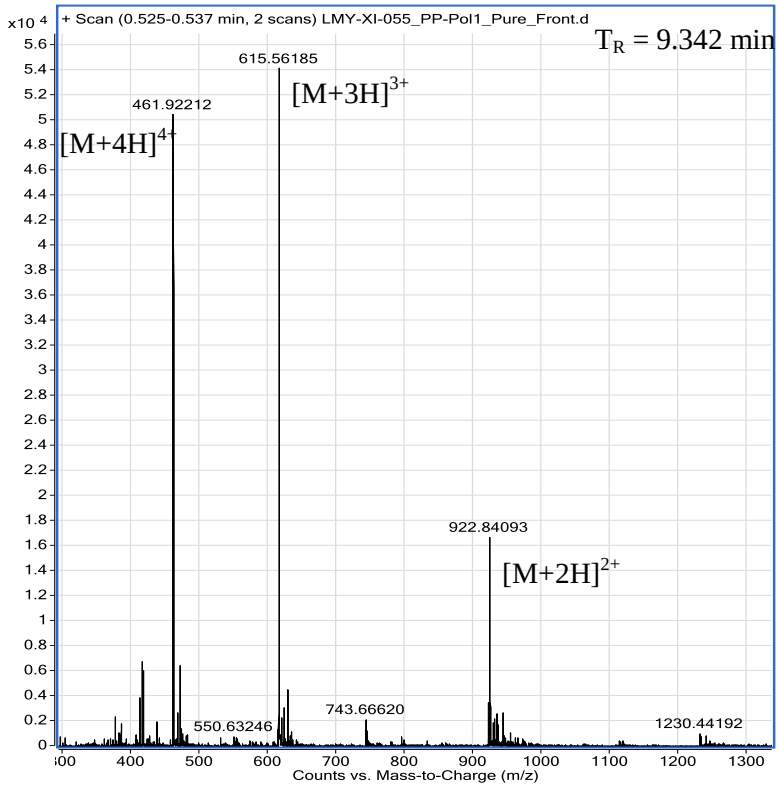


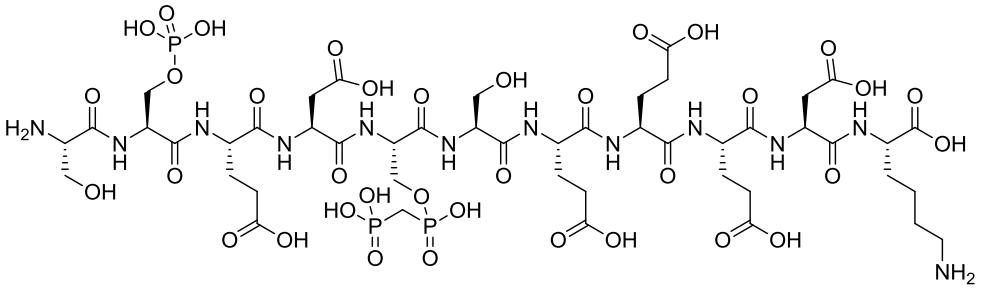




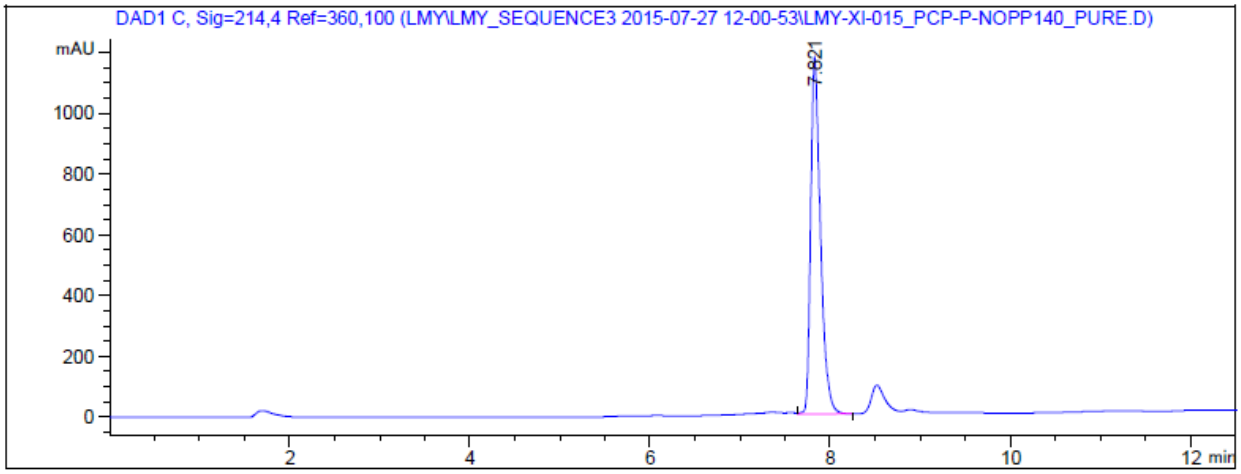
Ser-His-His-Asp-Asp-Glu-Glu-Glu-Ser-[PyroPSer]-Glu-Lys-Lys-Lys (PP-9)

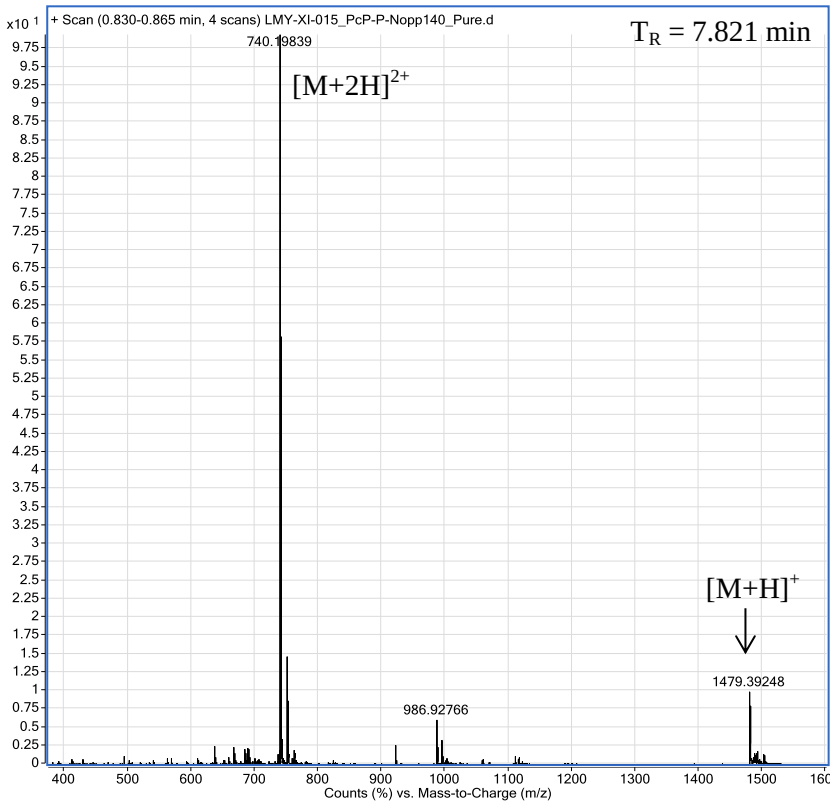


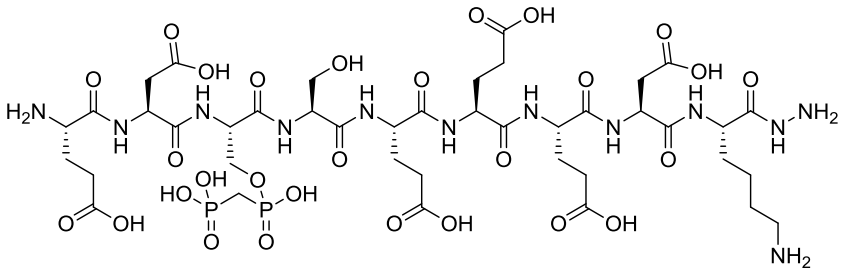




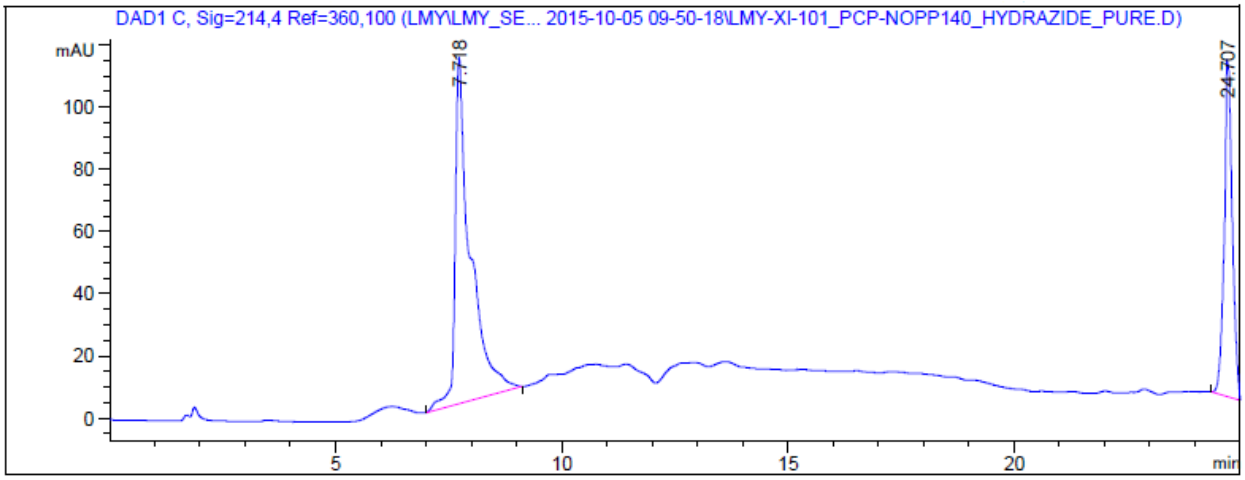
Ser-[PSer]-Glu-Asp-[PcPSer]-Ser-Glu-Glu-Glu-Asp-Lys (PCP-10)

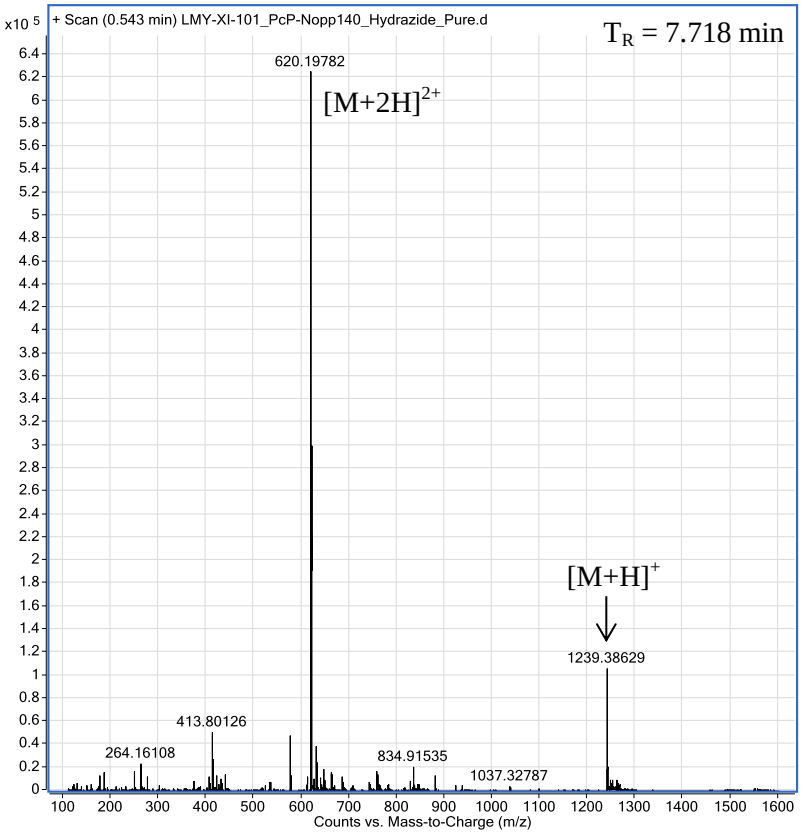


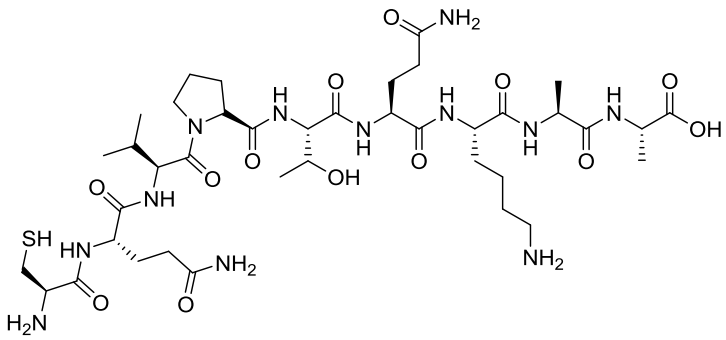




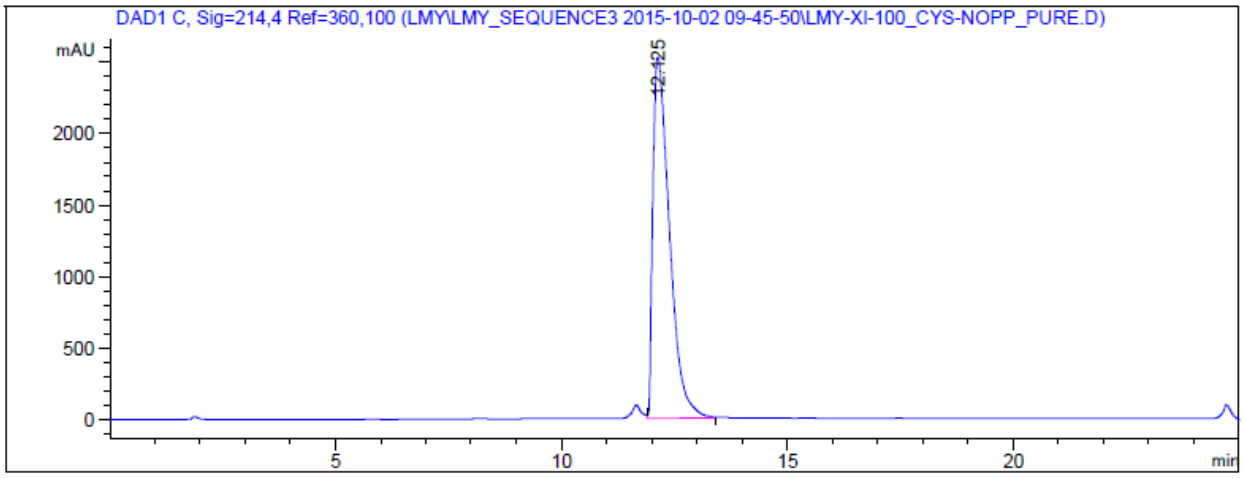
Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-NH-NH_2 (11)

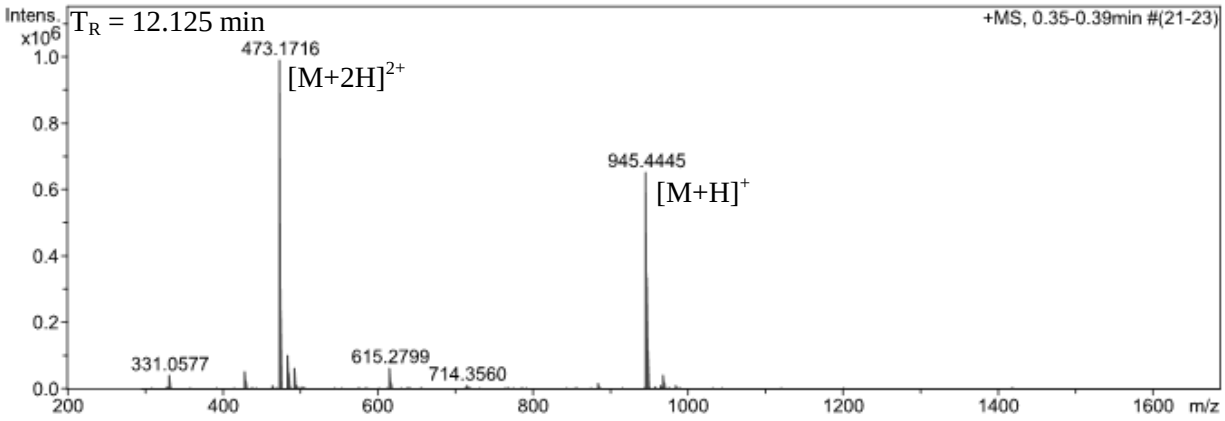


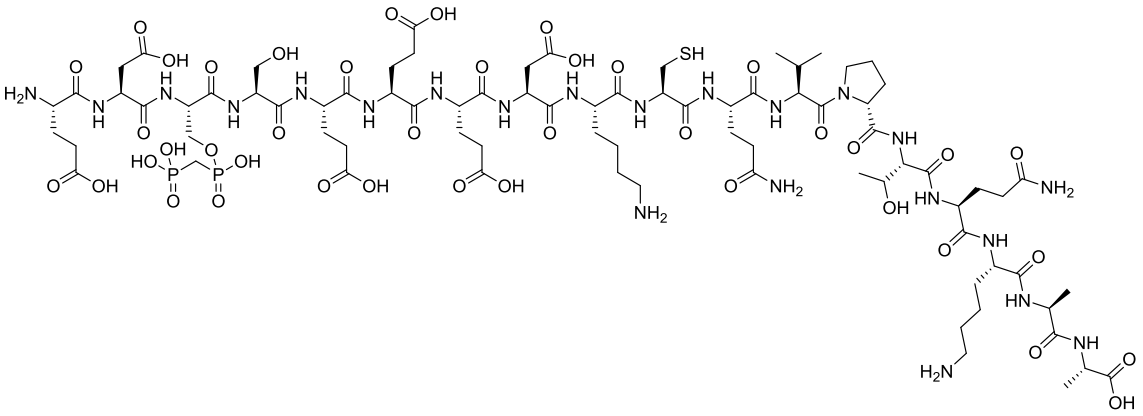




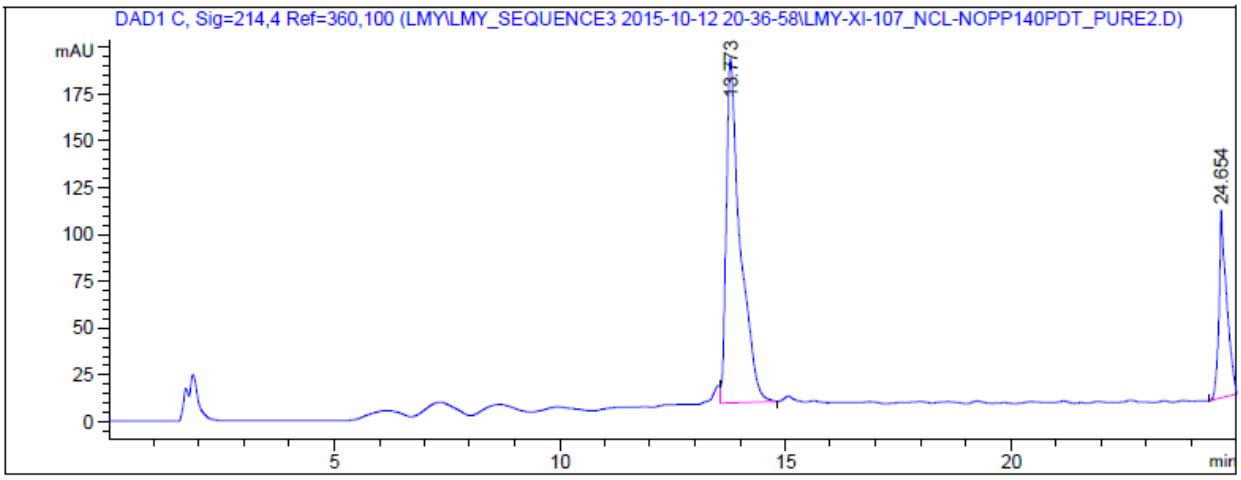
Cys-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (12)

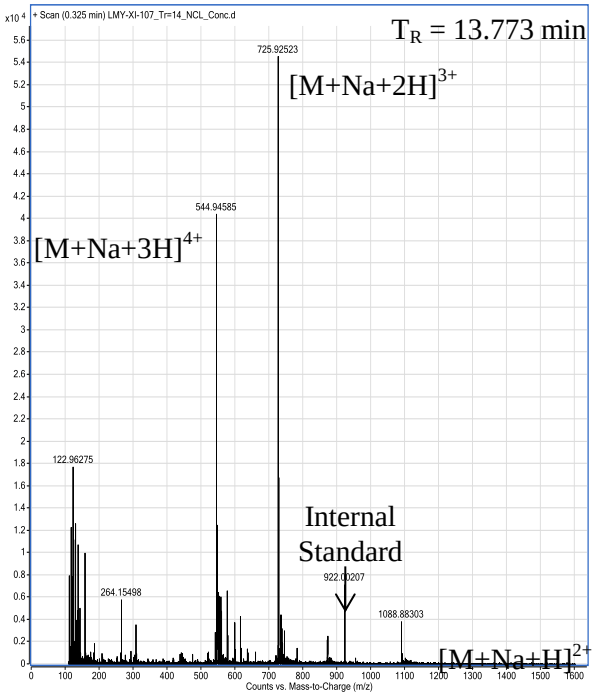


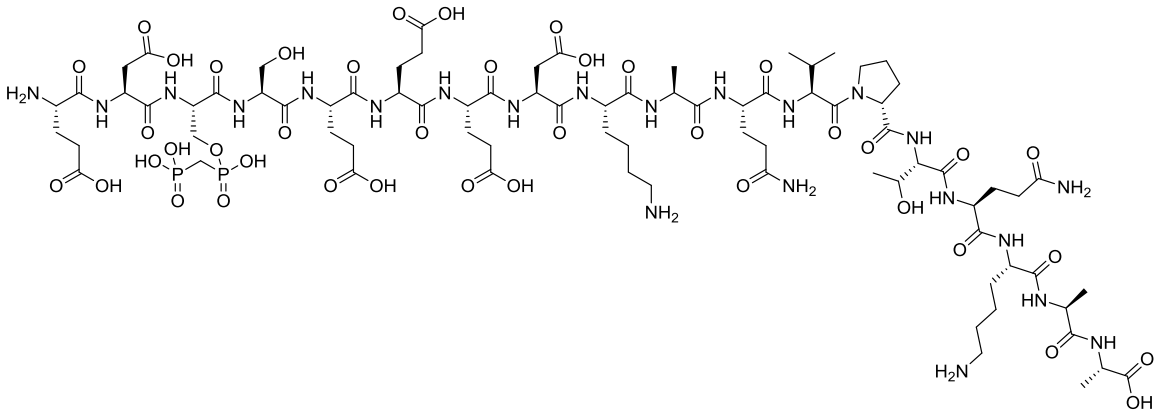




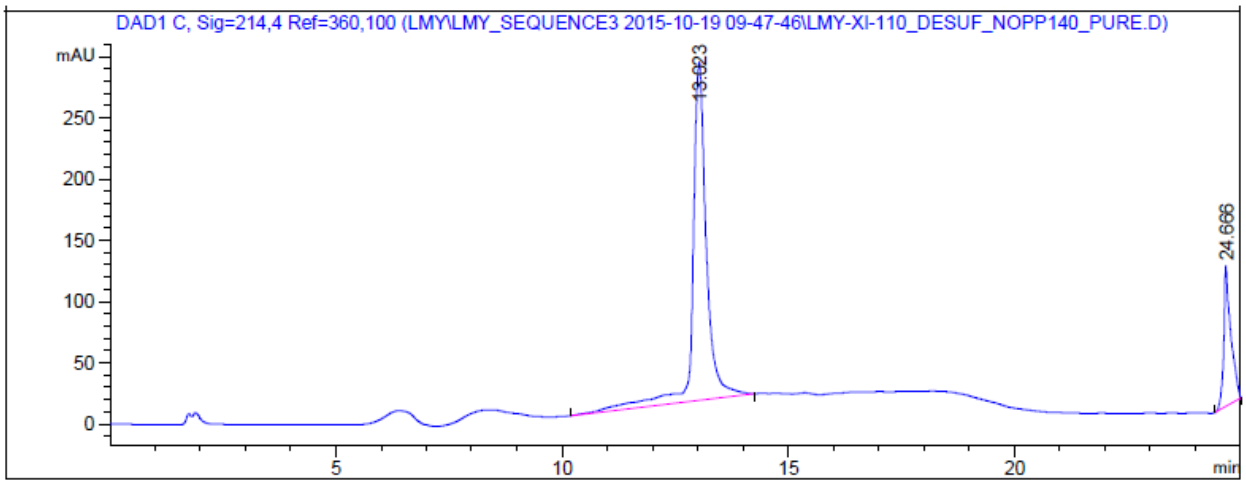
Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-Cys-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (13)

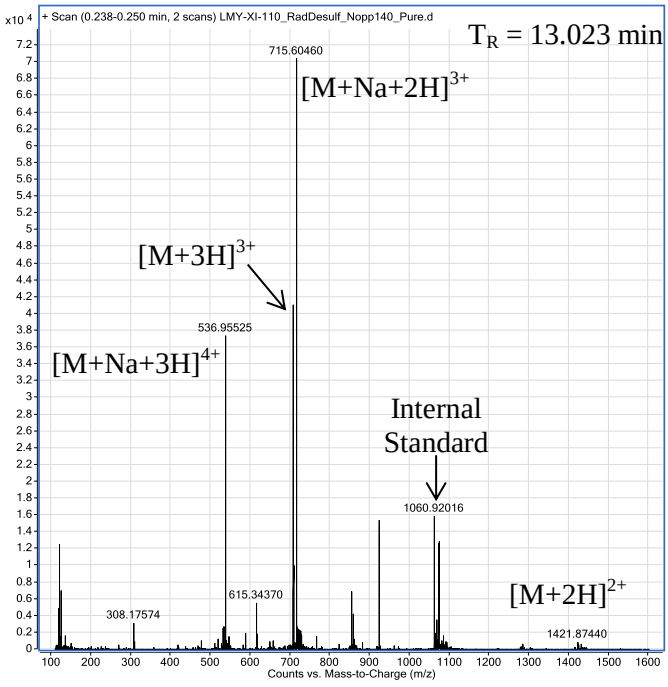




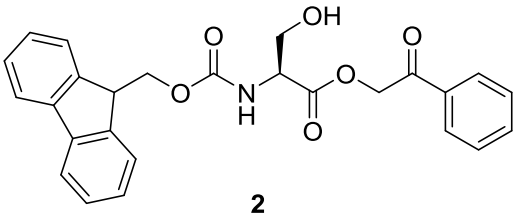


Glu-Asp-[PCP-Ser]-Ser-Glu-Glu-Glu-Asp-Lys-Ala-Gln-Val-Pro-Thr-Gln-Lys-Ala-Ala (14)





VIII. NMR Spectra

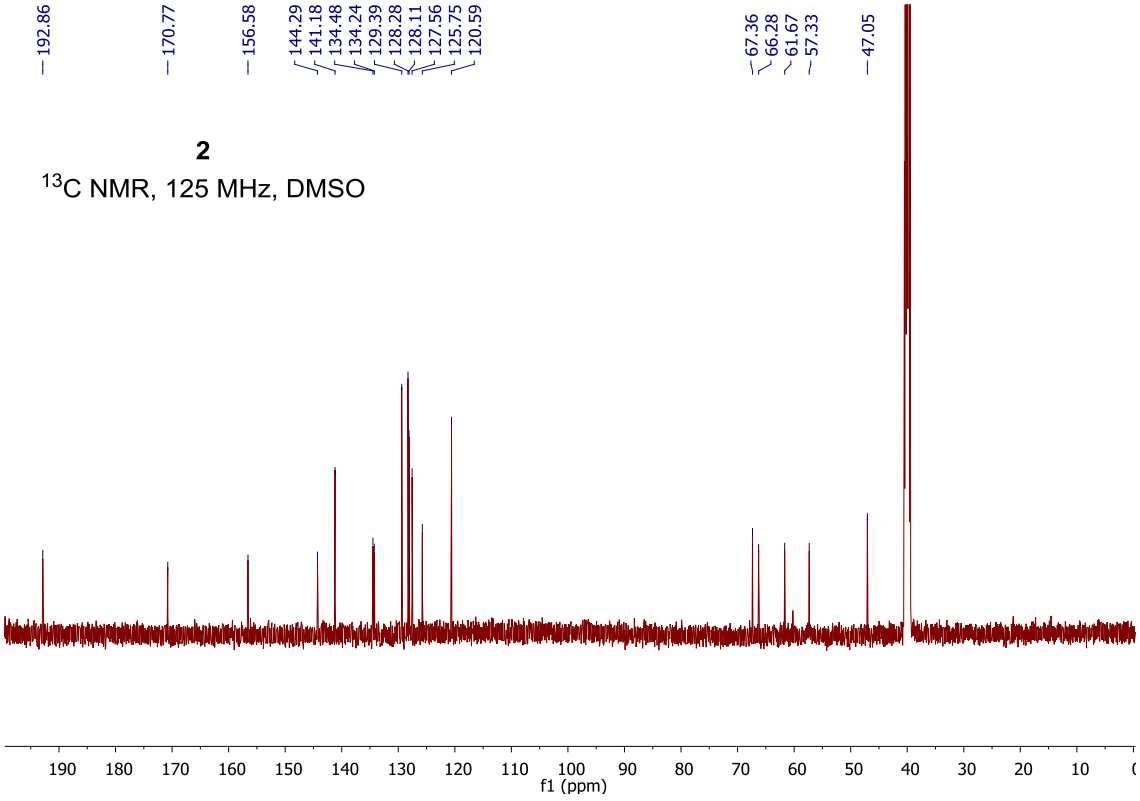


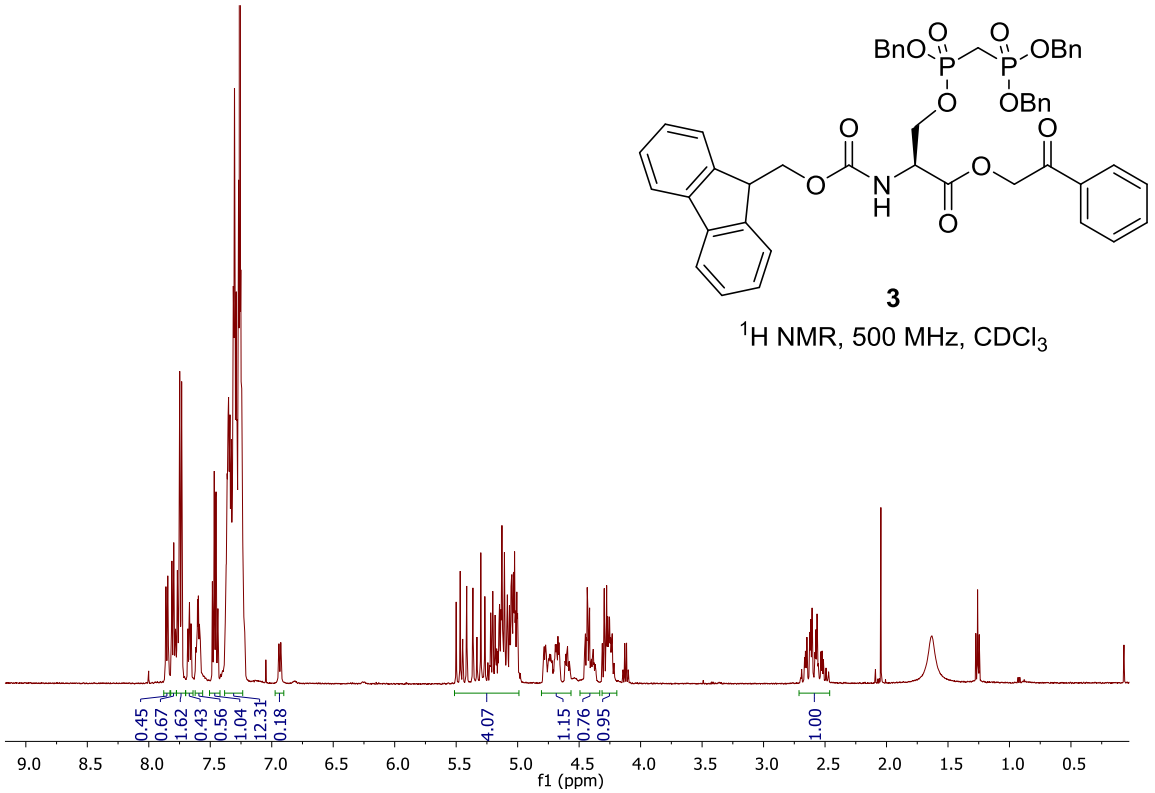
2

^{1}H NMR, 500 MHz, DMSO

2

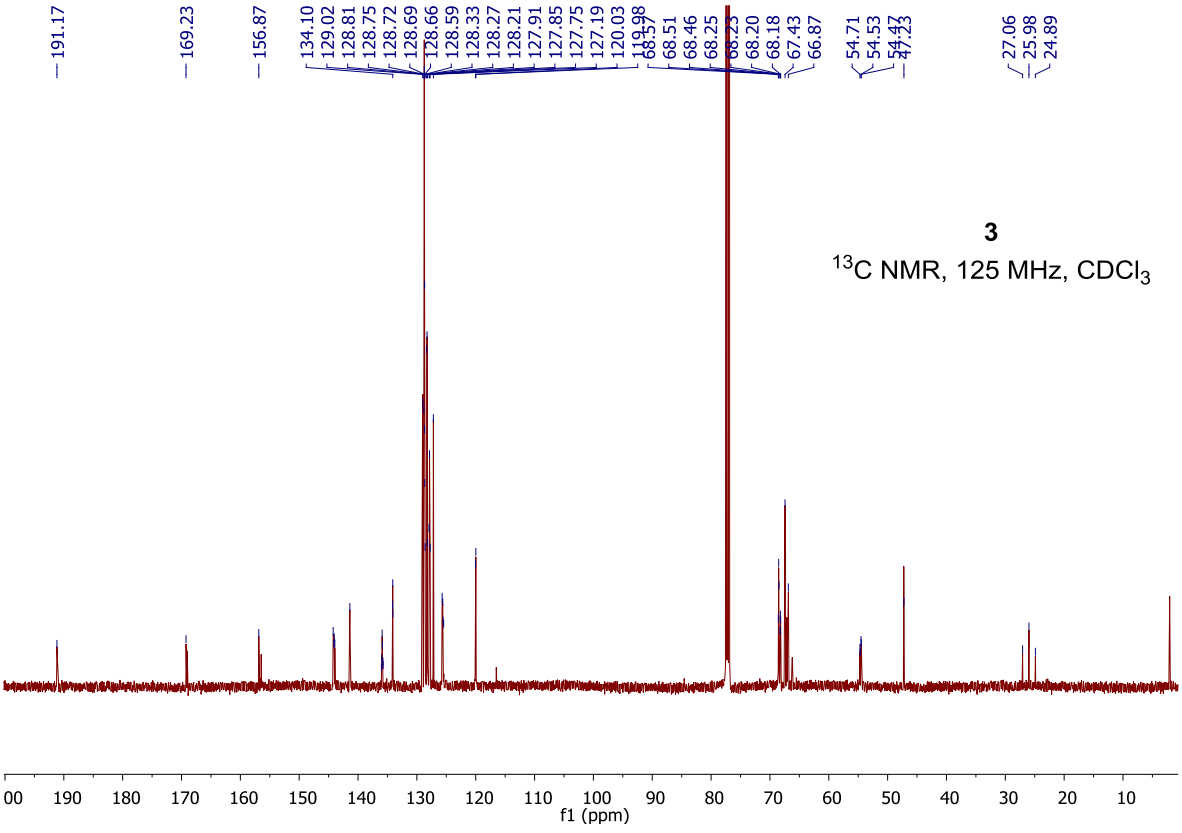
^{13}C NMR, 125 MHz, DMSO





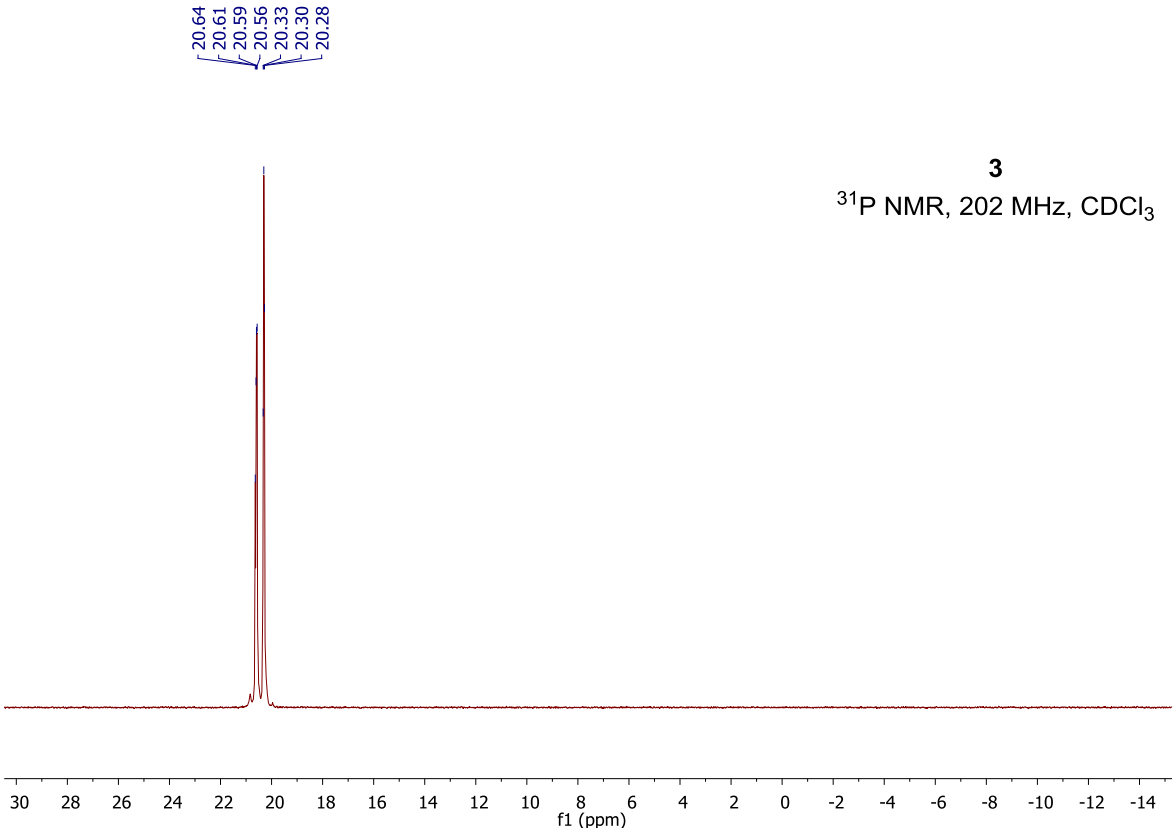
3

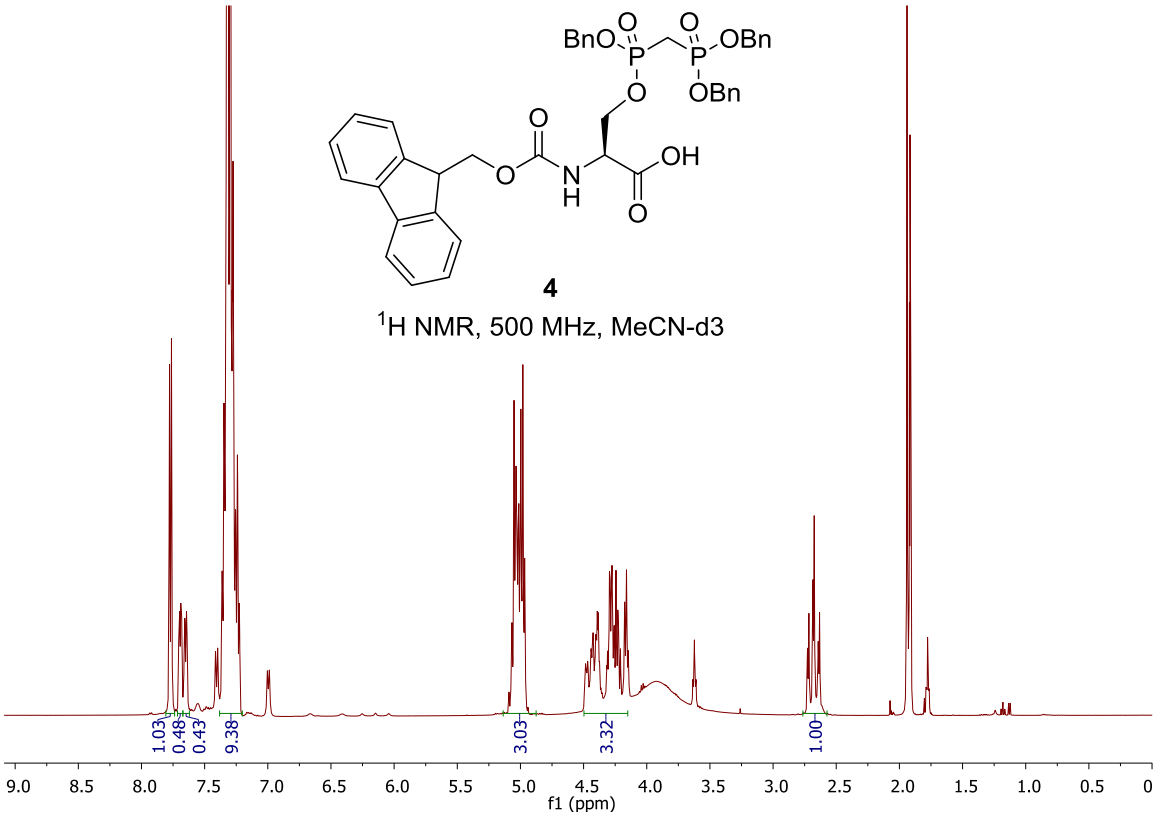
^{1}H NMR, 500 MHz, $CDCl_3$



3

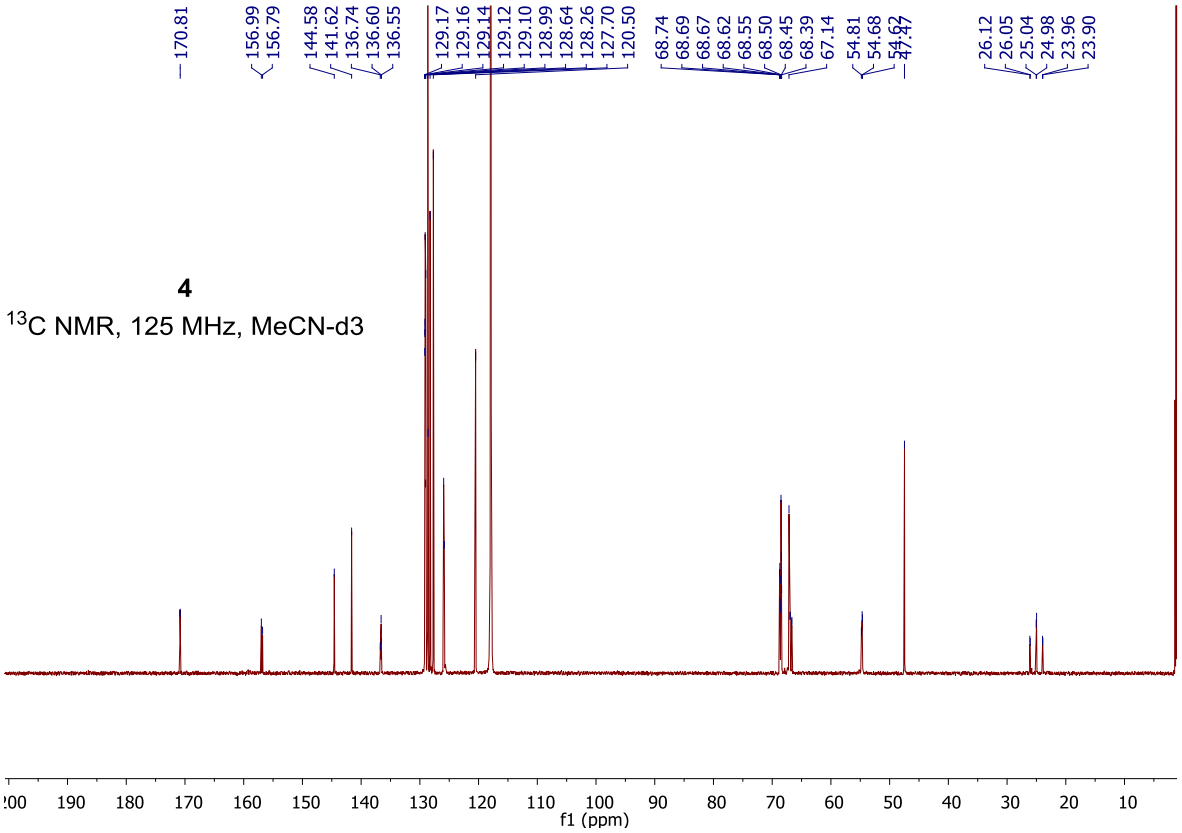
^{13}C NMR, 125 MHz, $CDCl_3$

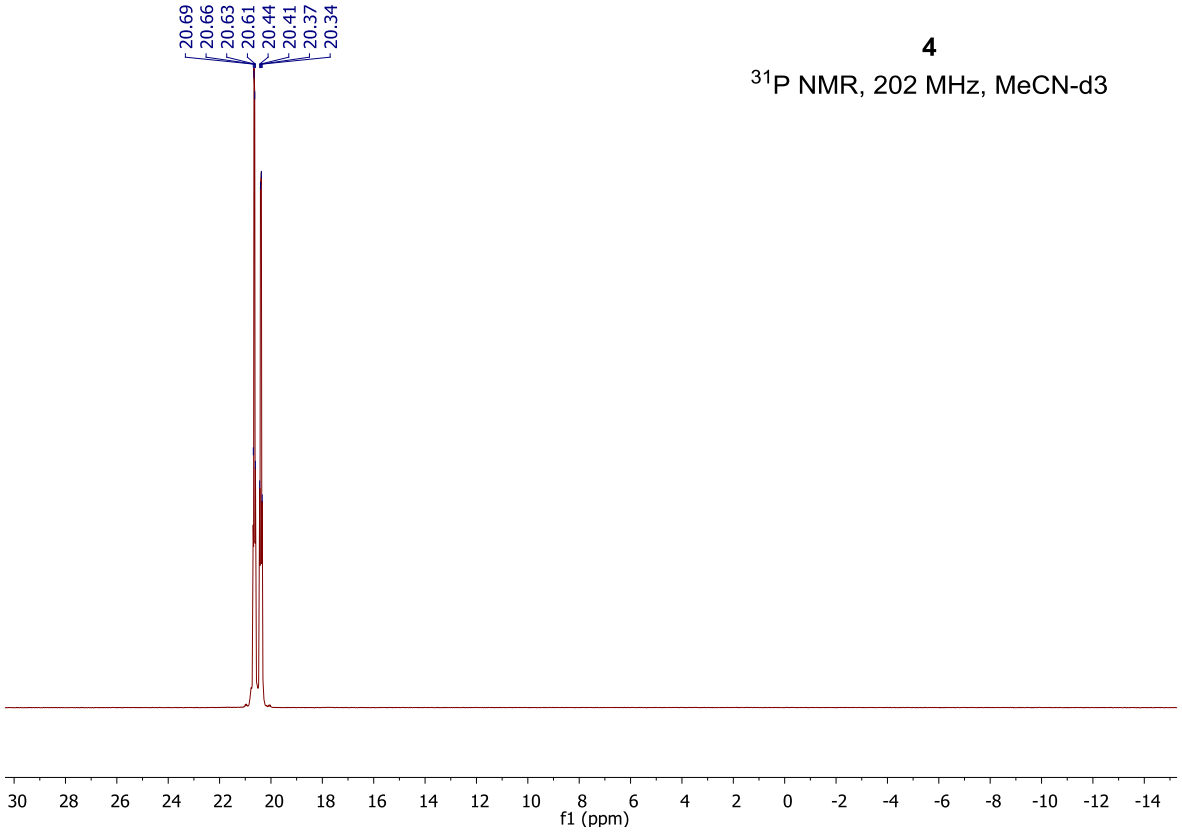

20.64
20.61
20.59
20.56
20.33
20.30
20.28
3
31P NMR, 202 MHz, CDCl3
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2 -4 -6 -8 -10 -12 -14
f1 (ppm)

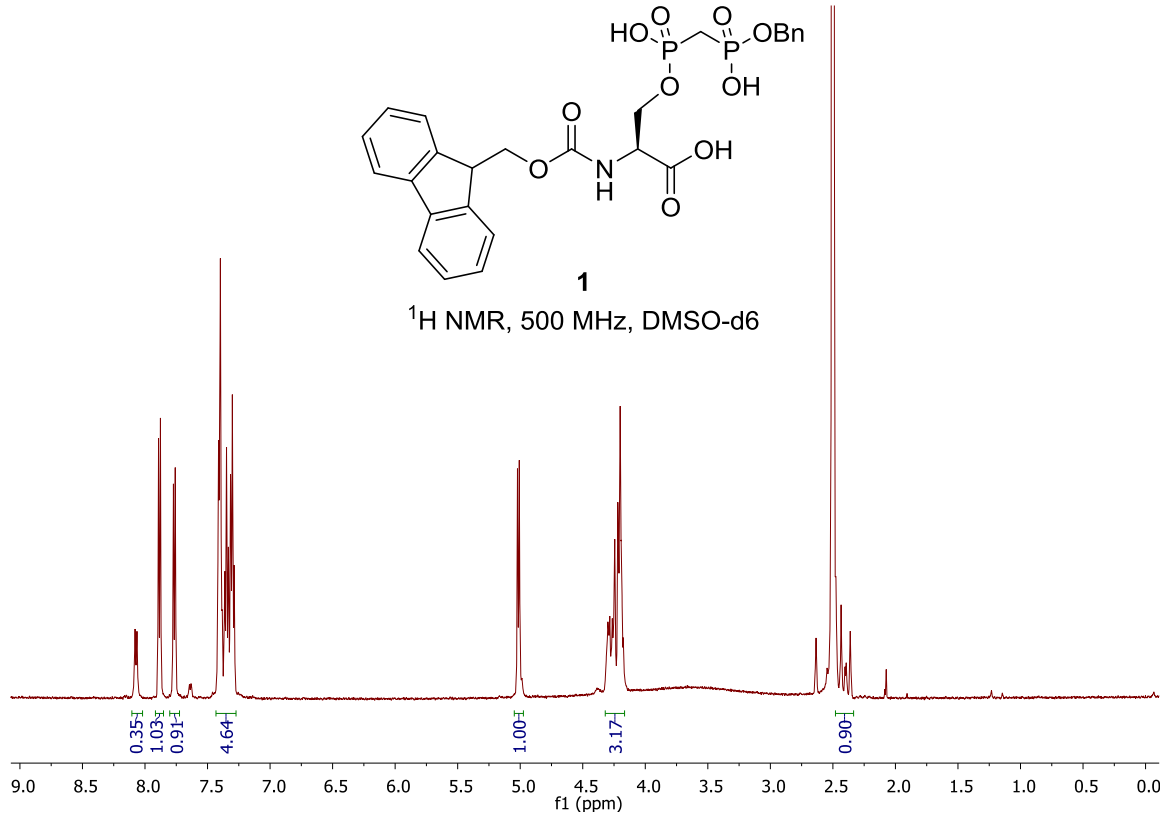


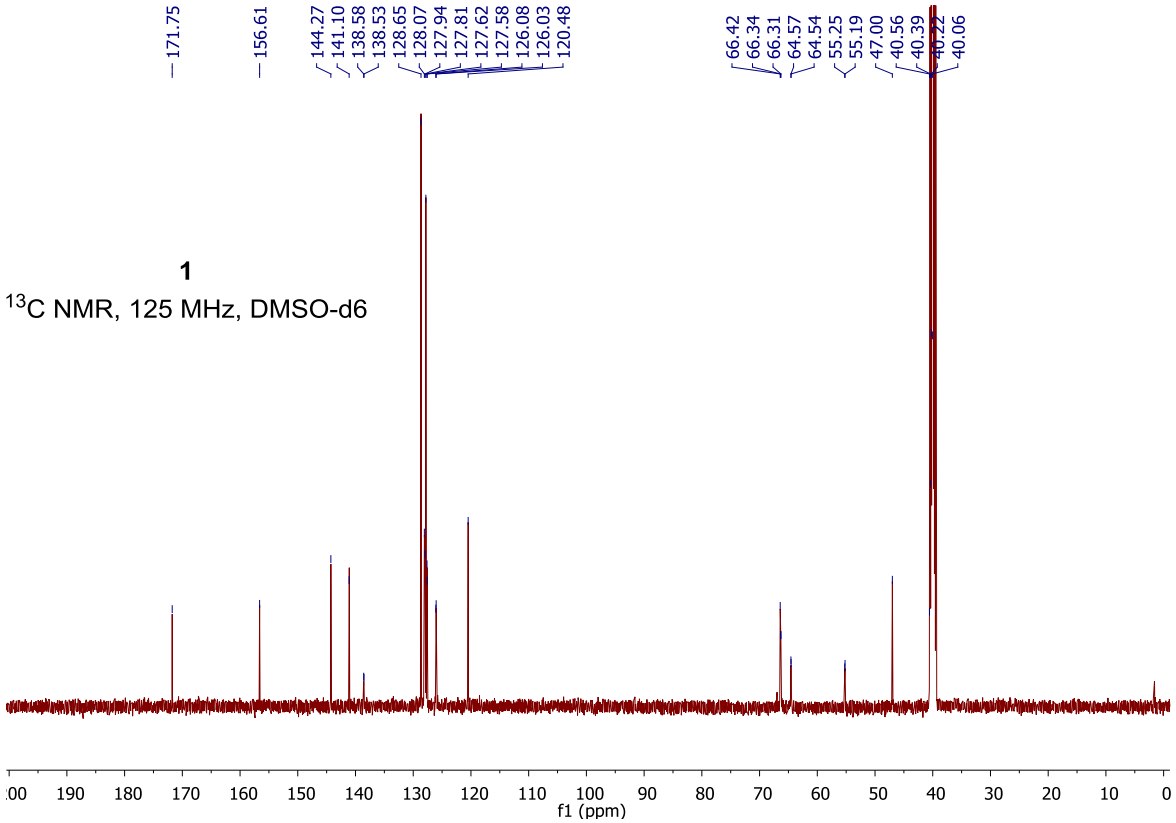
4

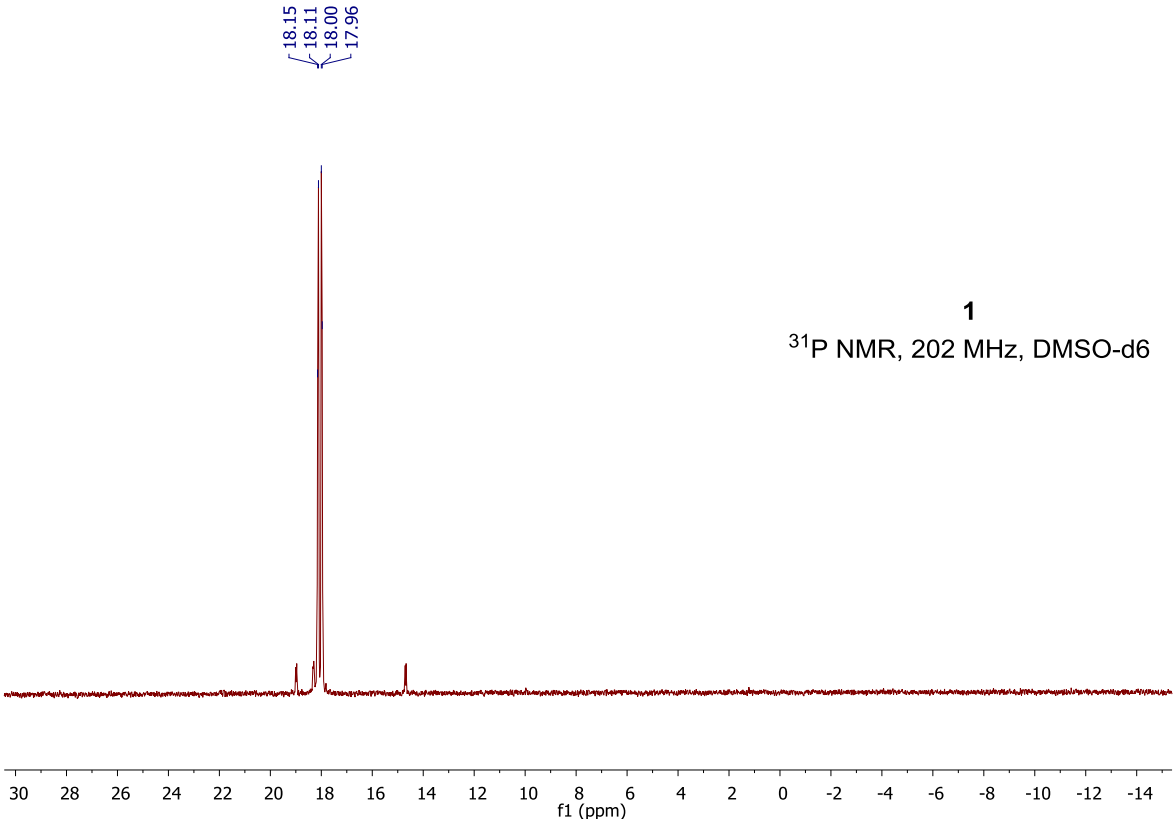
^{1}H NMR, 500 MHz, MeCN-d3

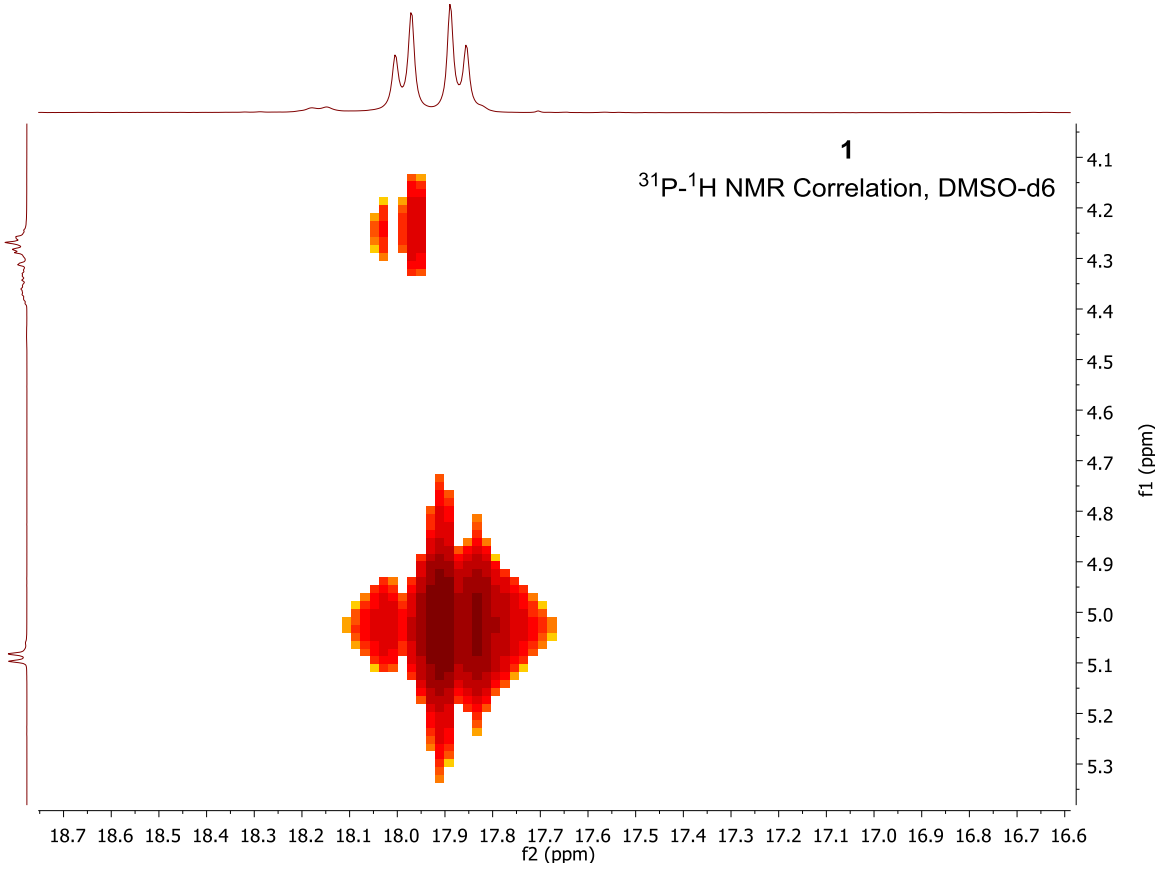

170.81
156.99
156.79
144.58
141.62
136.74
136.60
136.55
129.17
129.16
129.14
129.12
129.10
128.99
128.64
128.26
127.70
120.50
68.74
68.69
68.67
68.62
68.55
68.50
68.45
68.39
67.14
54.81
54.68
54.62
47.47
26.12
26.05
25.04
24.98
23.96
23.90
4
13C NMR, 125 MHz, MeCN-d3
f1 (ppm)

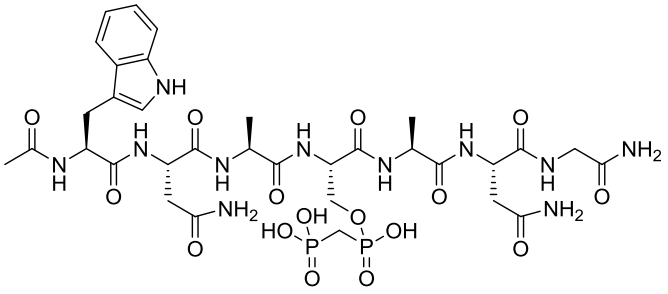

20.69
20.66
20.63
20.61
20.44
20.41
20.37
20.34
4
31P NMR, 202 MHz, MeCN-d3
f1 (ppm)


HO
O
P
O
O
P
OBn
O
OH
O
O
N
H
OH
O
1
1H NMR, 500 MHz, DMSO-d6
0.35
1.03
0.91
4.64
1.00
3.17
0.90
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


171.75
156.61
144.27
141.10
138.58
138.53
128.65
128.07
127.94
127.81
127.62
127.58
126.08
126.03
120.48
66.42
66.34
66.31
64.57
64.54
55.25
55.19
47.00
40.56
40.39
40.22
40.06
1
13C NMR, 125 MHz, DMSO-d6
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


18.15
18.11
18.00
17.96
1
31P NMR, 202 MHz, DMSO-d6
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2 -4 -6 -8 -10 -12 -14
f1 (ppm)

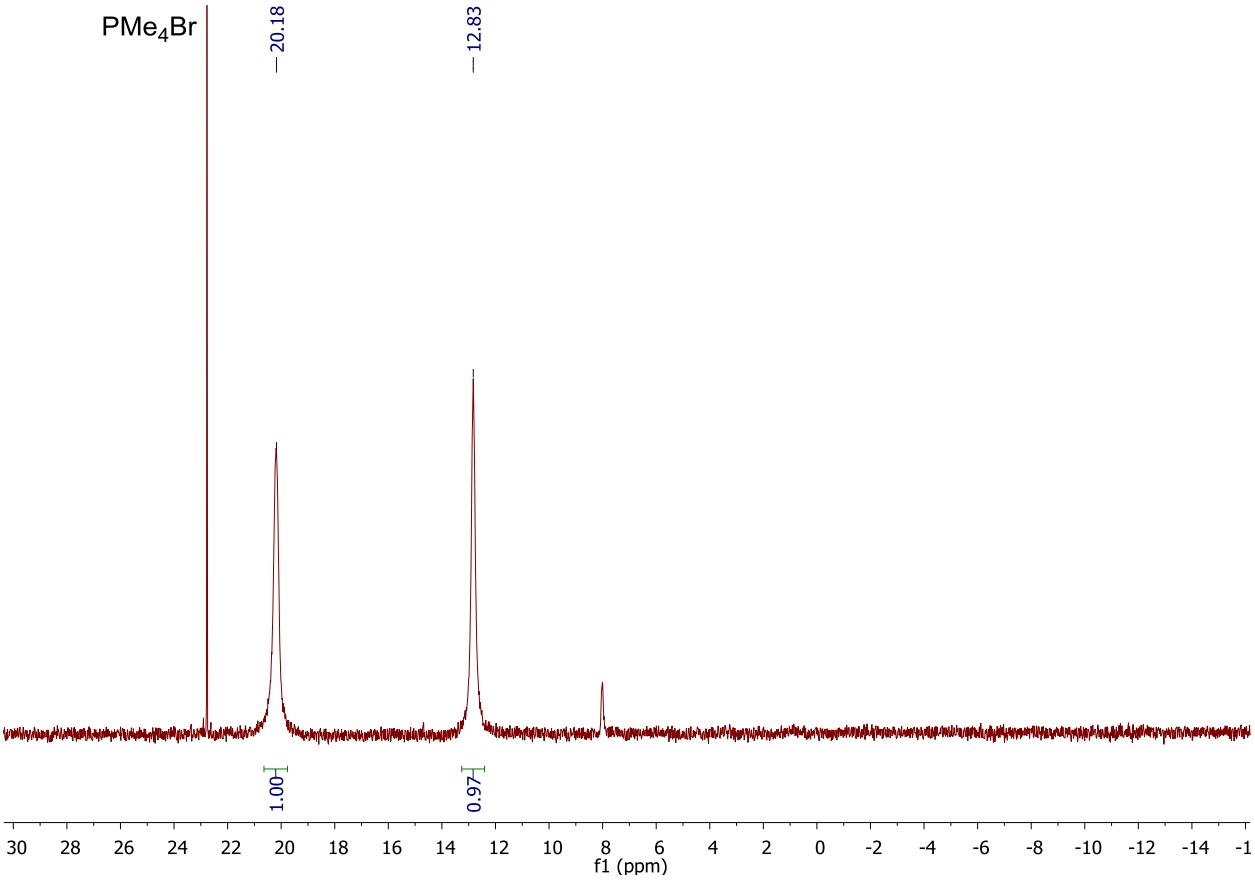

1
31P-1H NMR Correlation, DMSO-d6
4.1 4.2 4.3 4.4 4.5 4.6 4.7 4.8 4.9 5.0 5.1 5.2 5.3
f1 (ppm)
18.7 18.6 18.5 18.4 18.3 18.2 18.1 18.0 17.9 17.8 17.7 17.6 17.5 17.4 17.3 17.2 17.1 17.0 16.9 16.8 16.7 16.6
f2 (ppm)

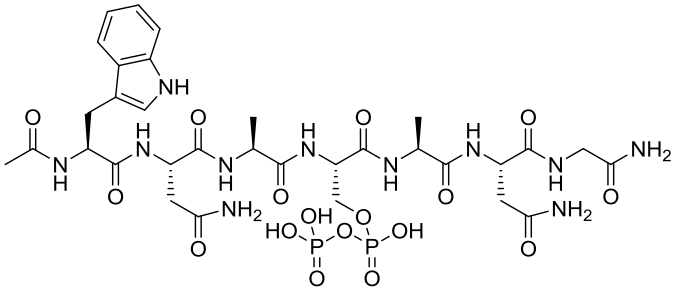


PCP-5

^{31}P NMR, 202 Hz, H_2O

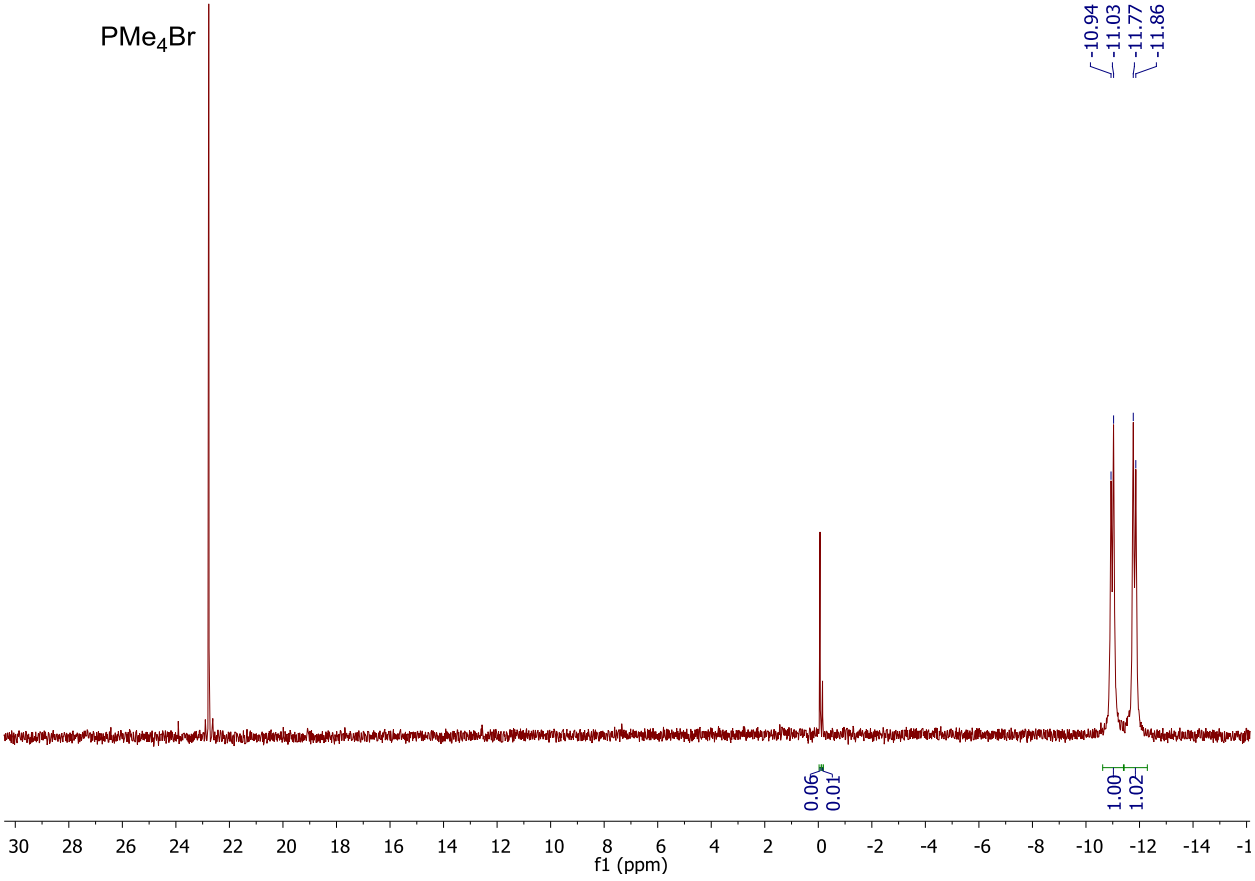
D_2O Lock

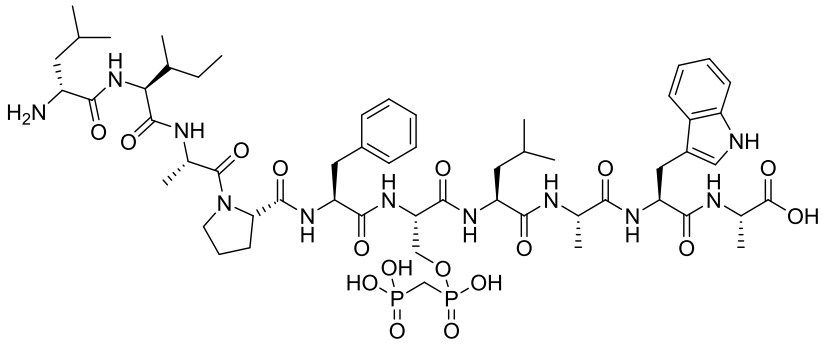




PP-5

^{31}P NMR, 202 Hz, D_2O

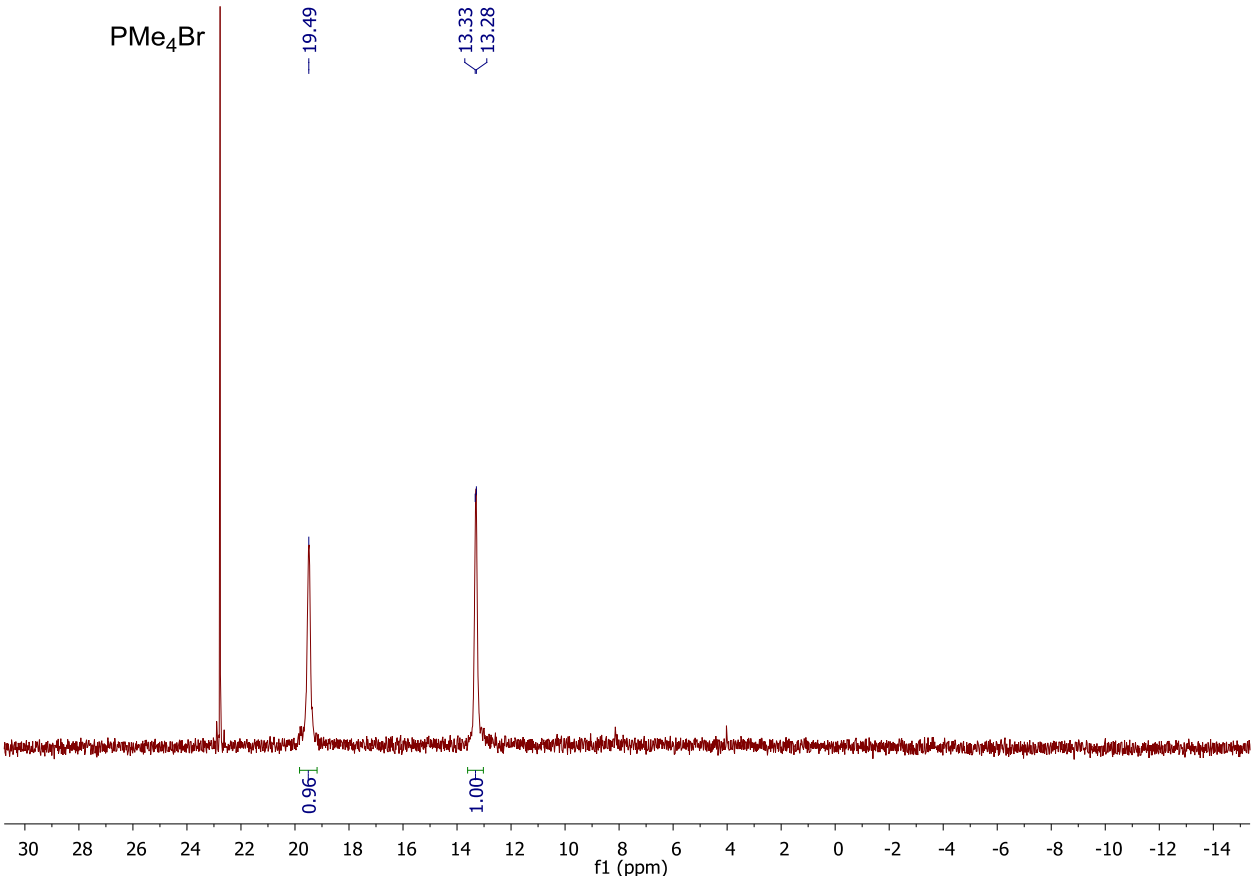


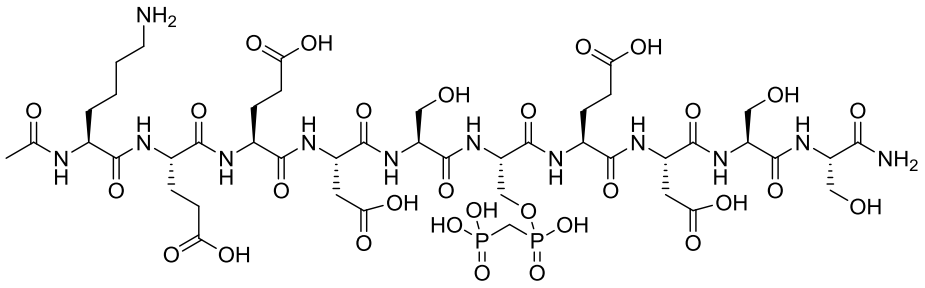


PCP-6

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

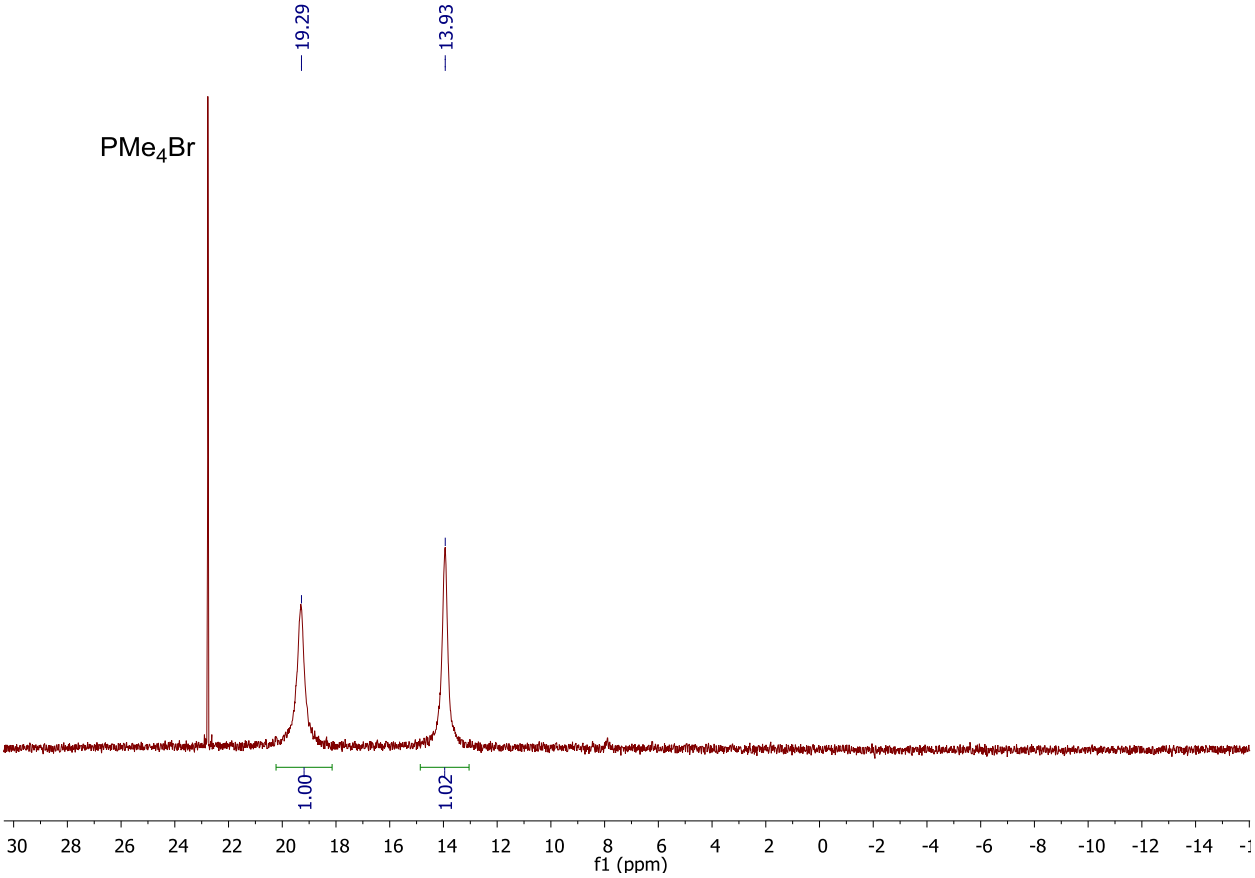


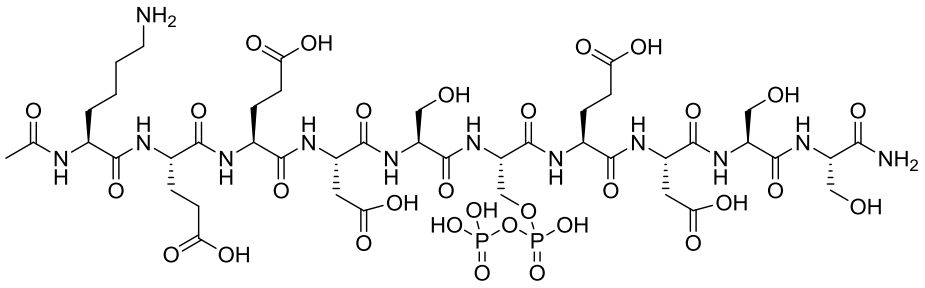


PCP-7

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

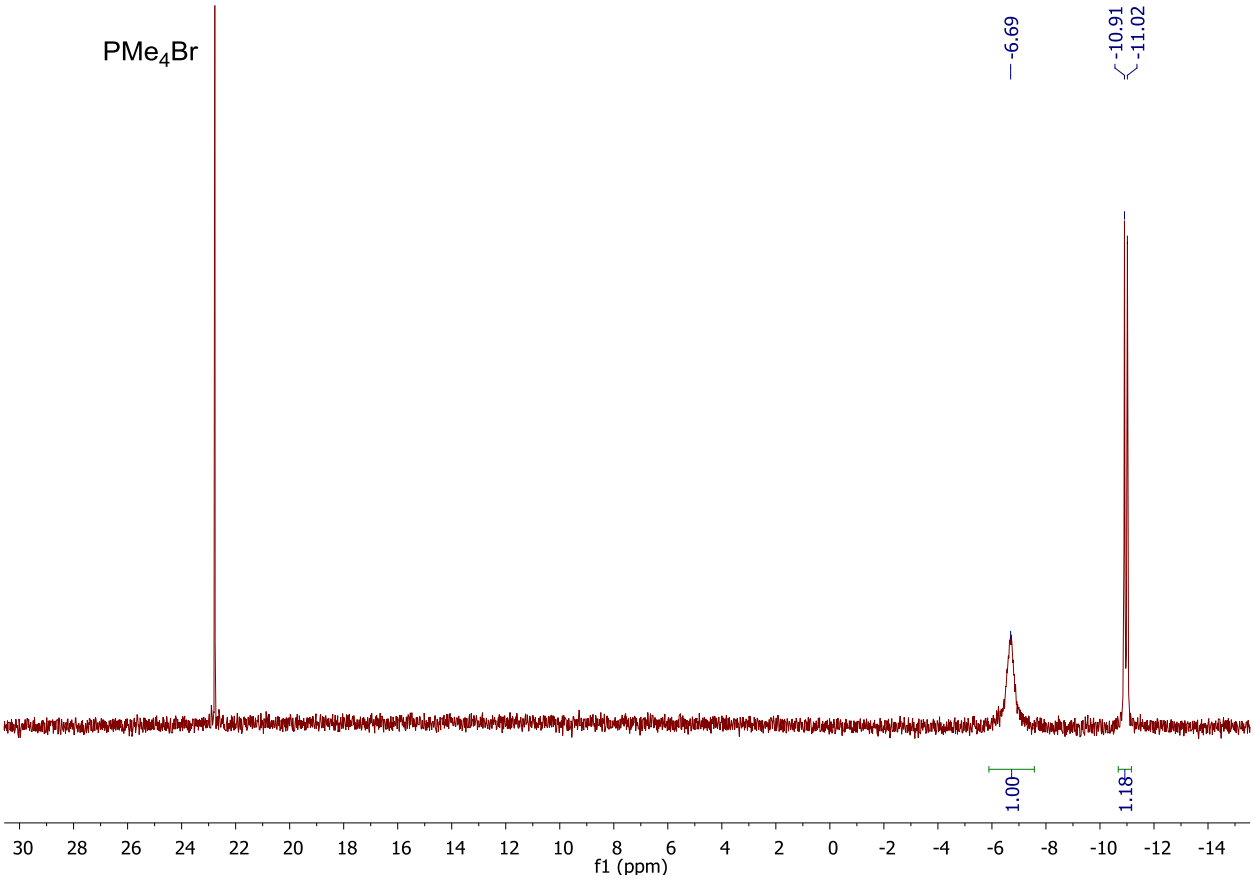


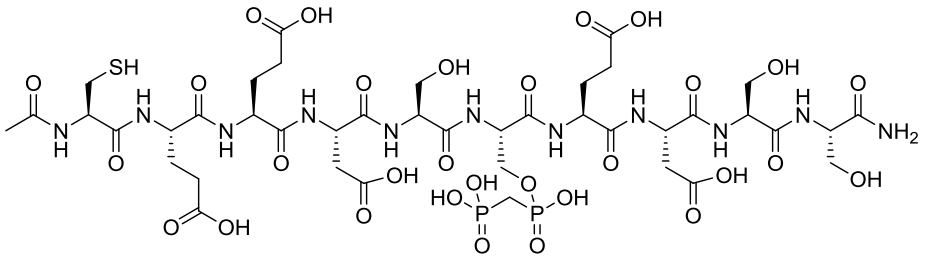


PP-7

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

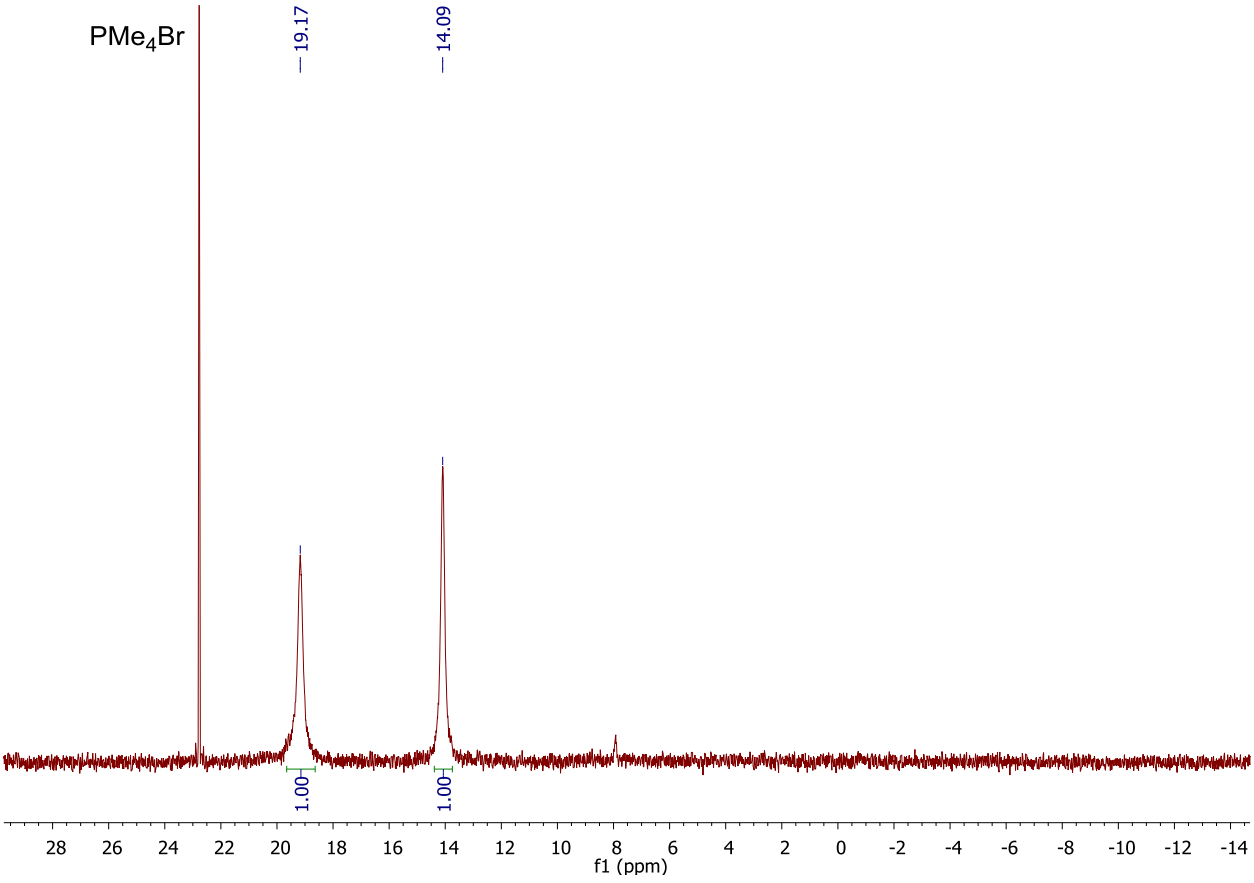


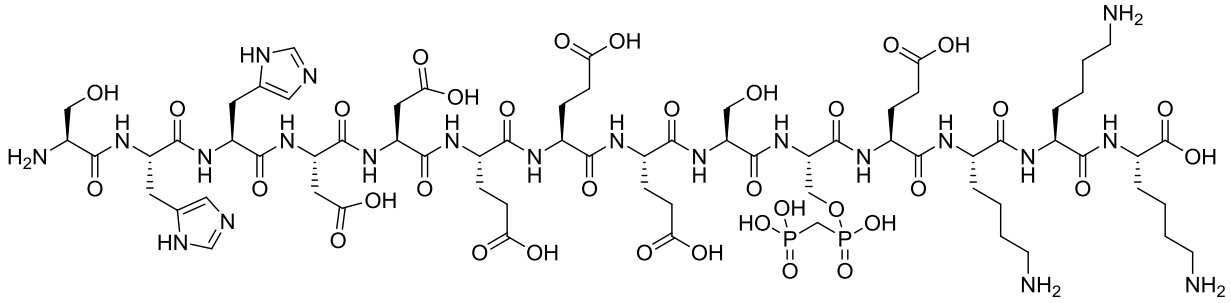


PCP-8

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

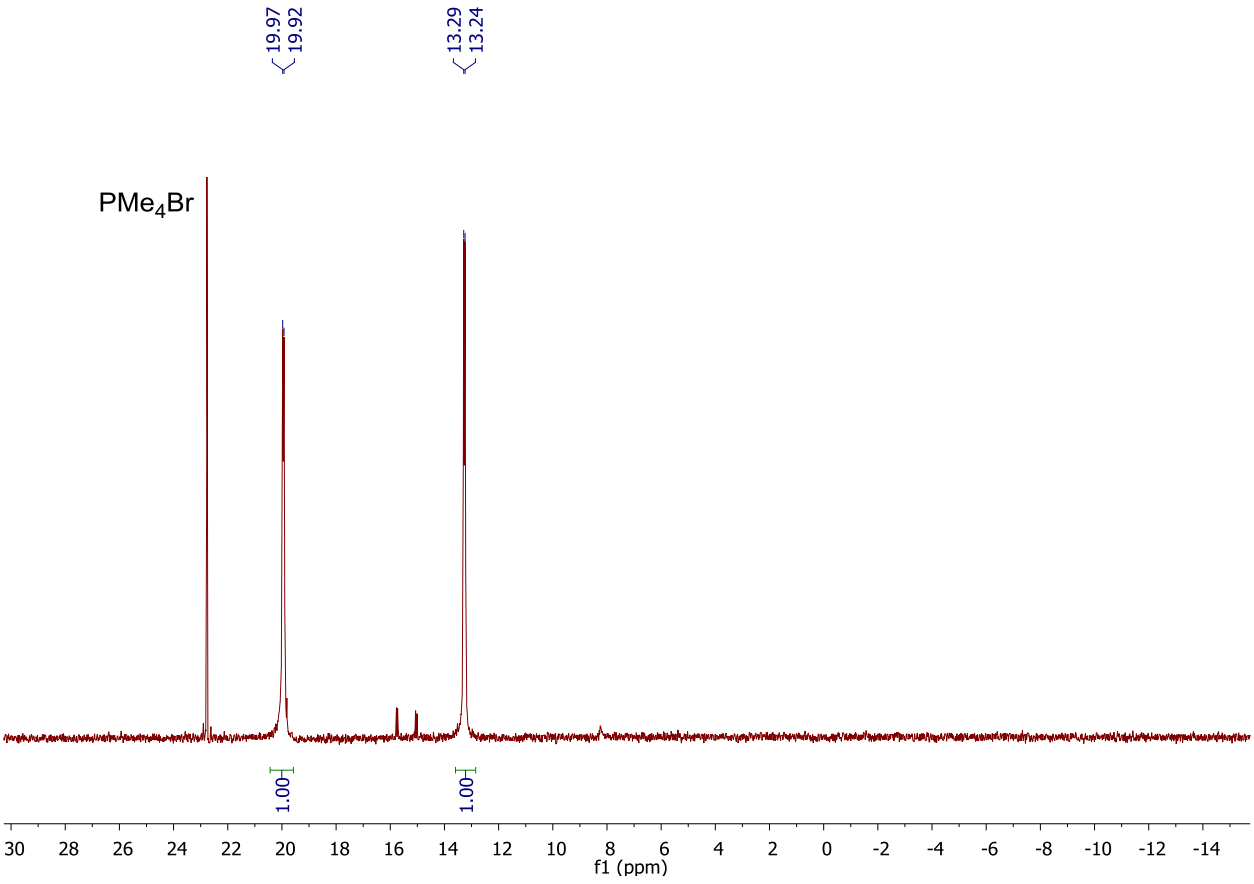


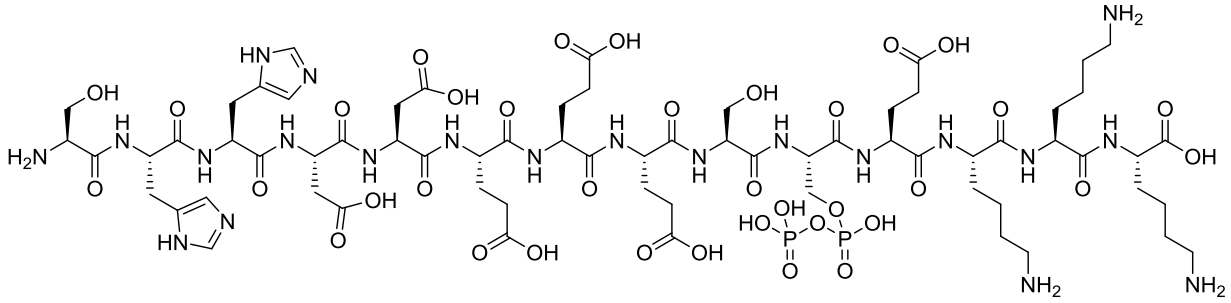


PCP-9

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

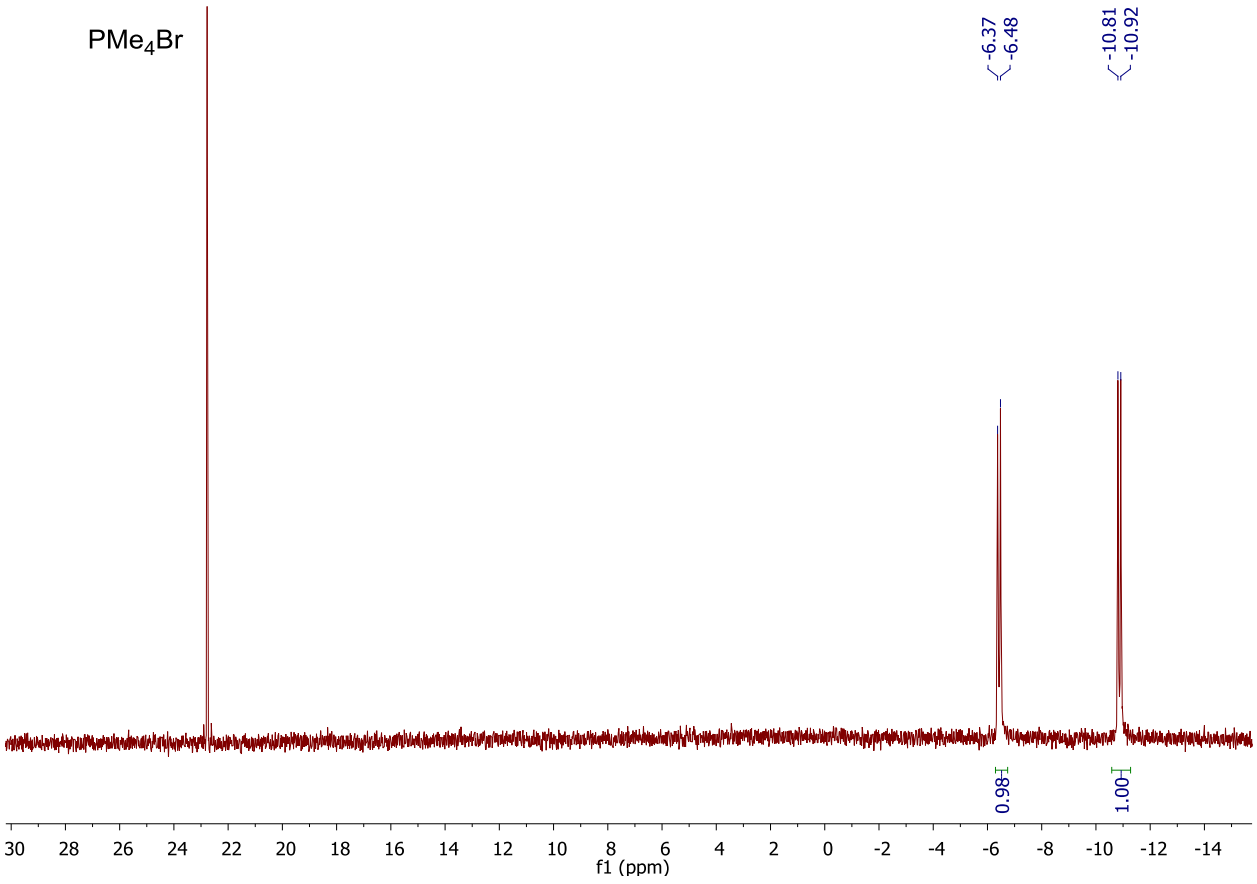


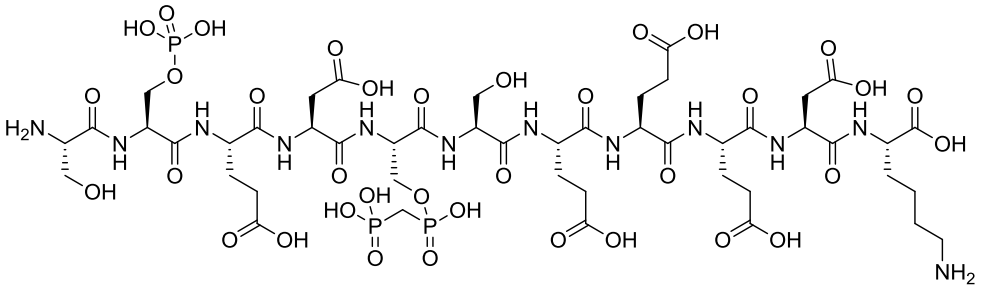


PP-9

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

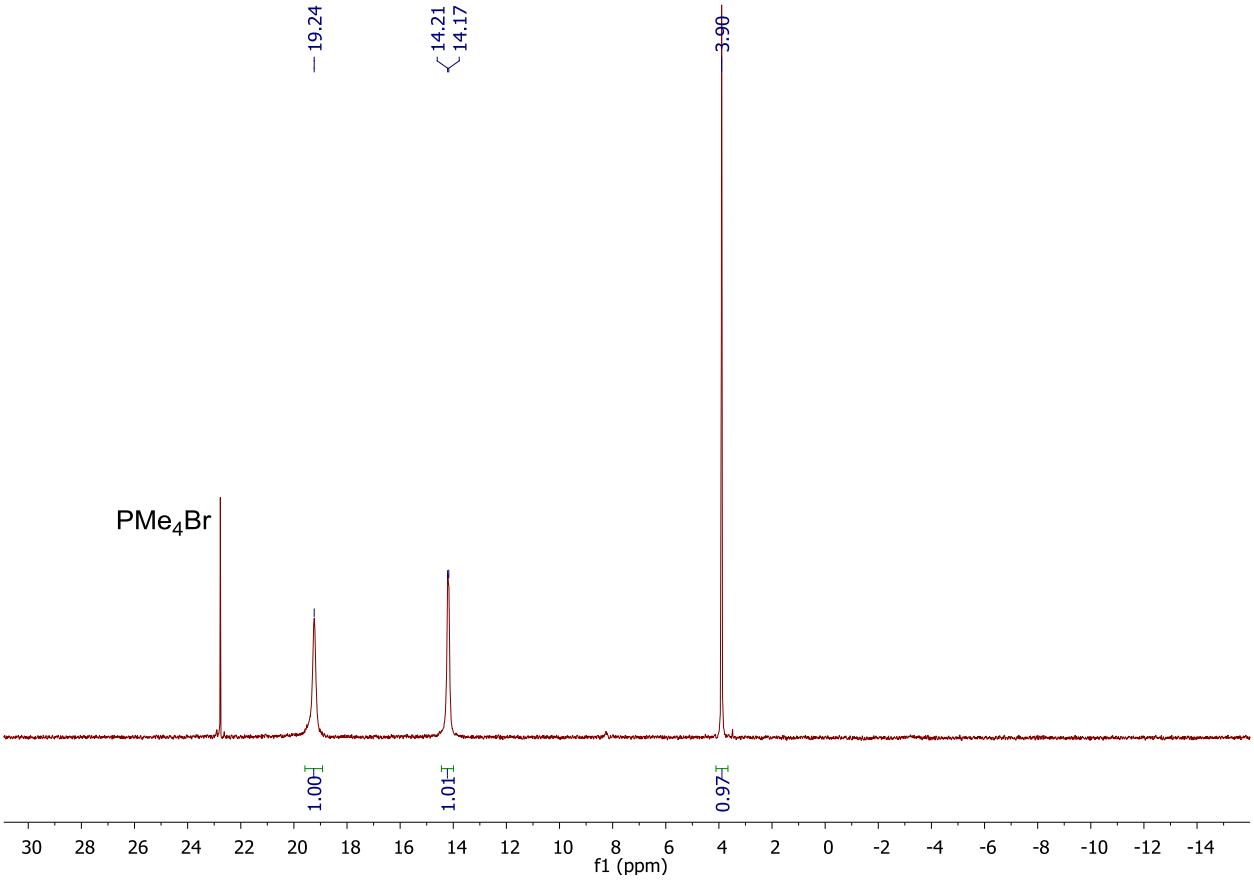


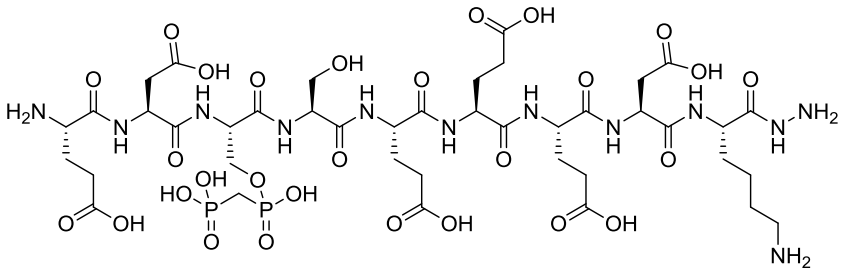


PCP-10

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

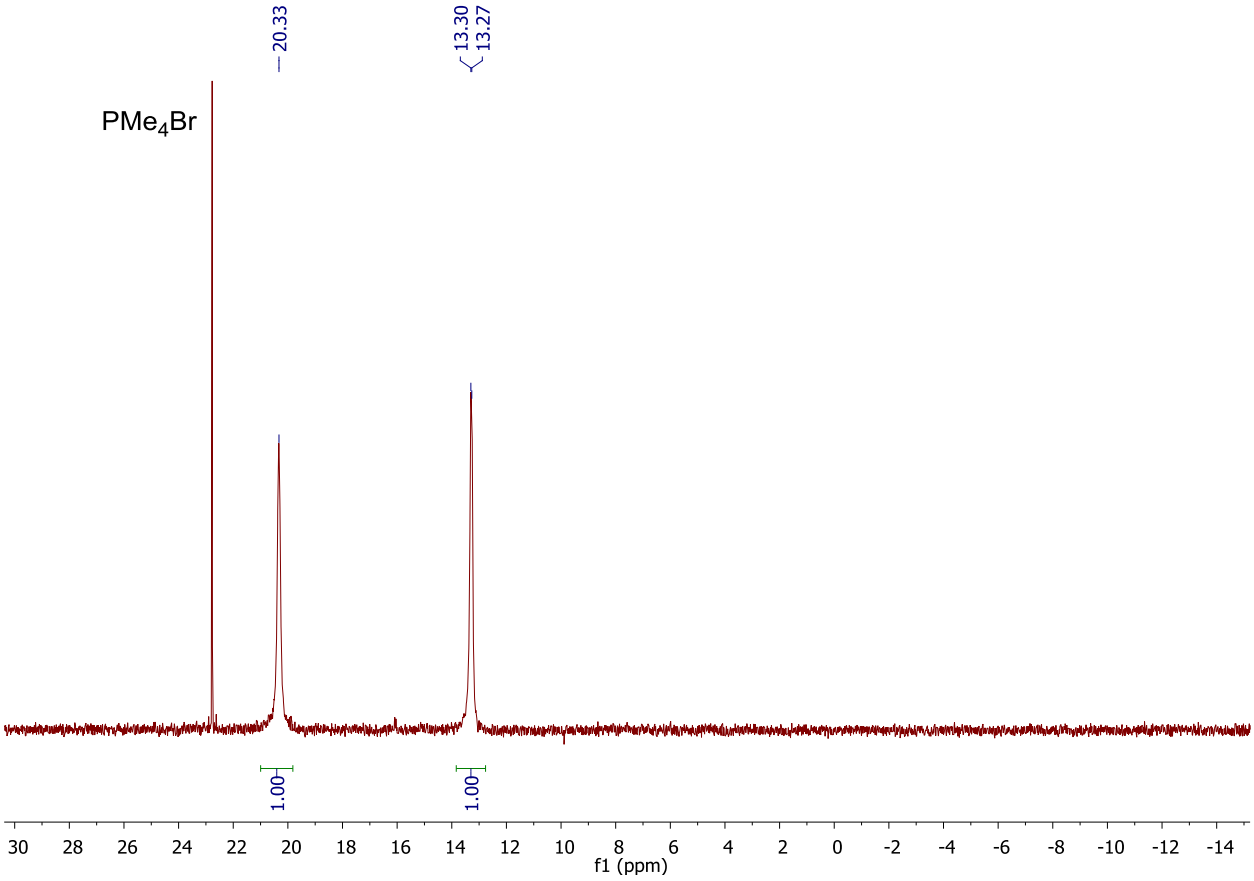


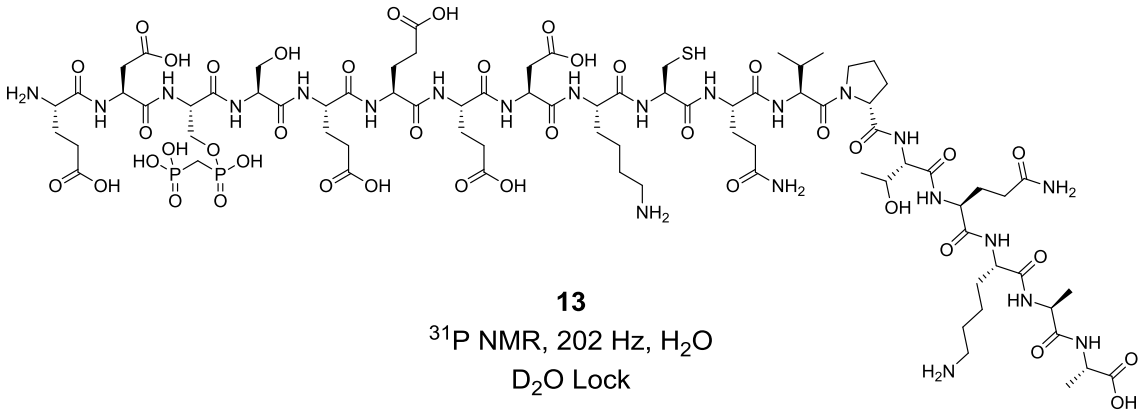


11

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

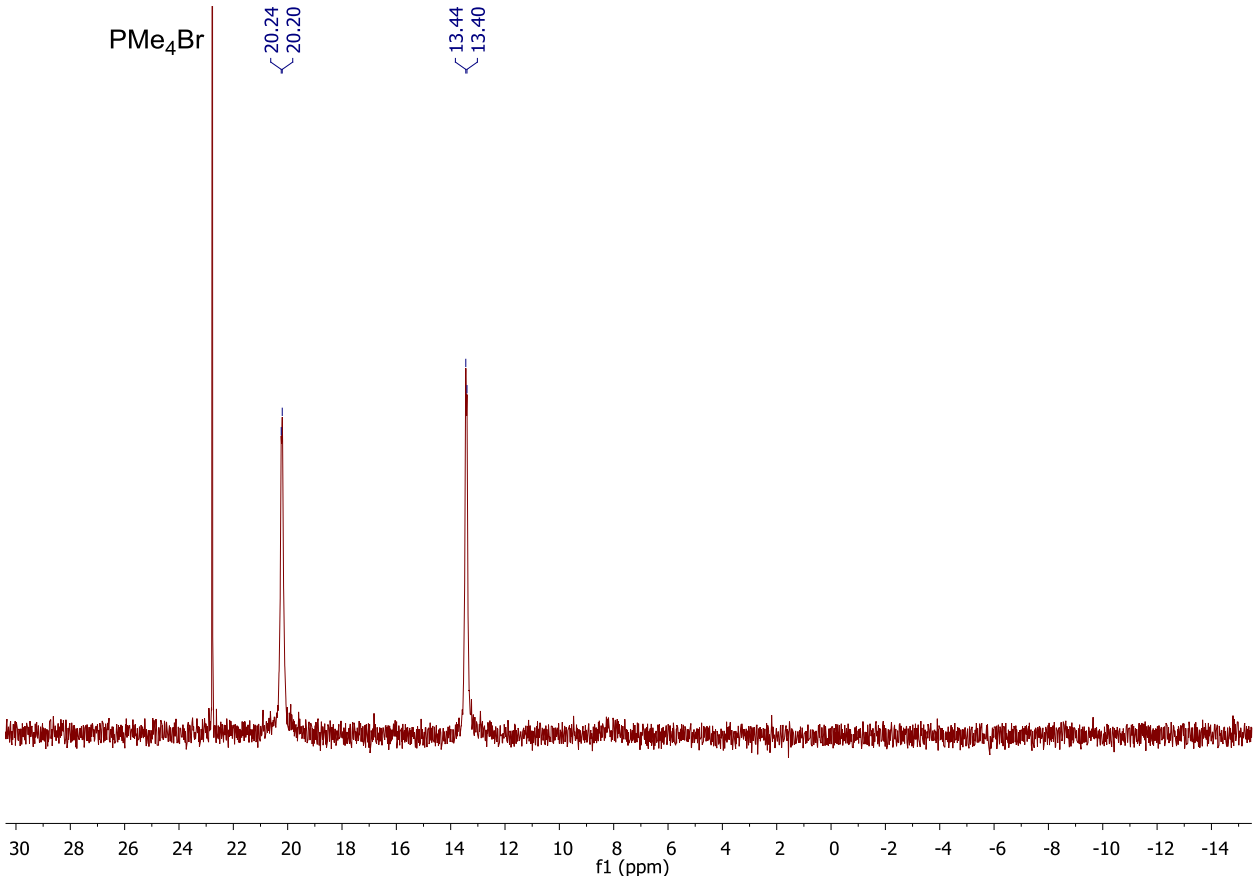


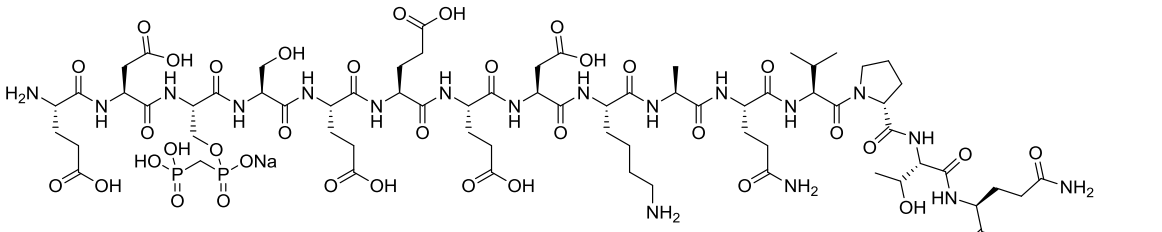


13

^{31}P NMR, 202 Hz, H_2O

D_2O Lock

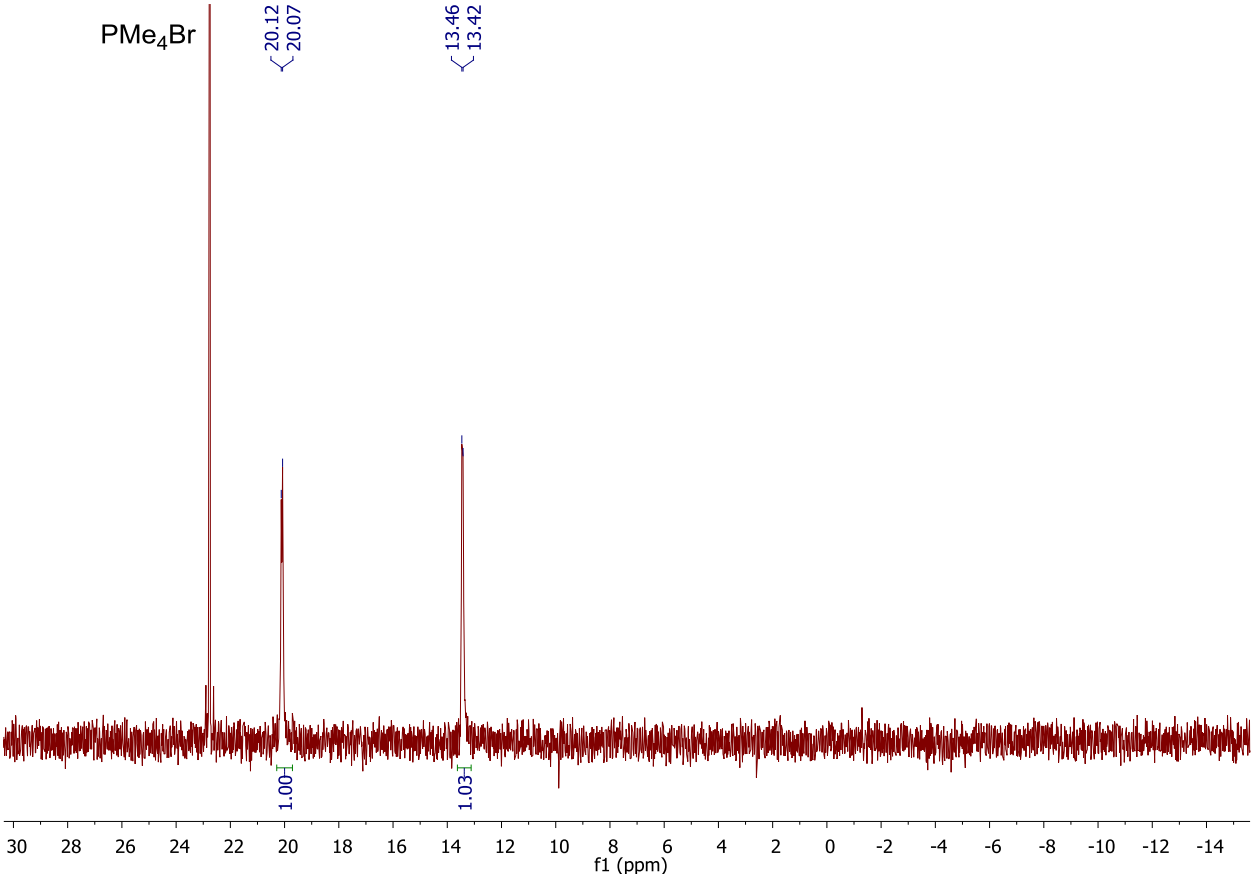




14

^{31}P NMR, 202 Hz, H_2O

D_2O Lock



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