

A targeted approach for the synthesis of multi-phosphorylated peptides: new tool for studying the role of phosphorylation patterns in proteins

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1. Materials

All solvents and reagents were used as supplied. (1-[Bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxid hexafluorophosphate)(HATU), Dimethylformamide (DMF) (peptide synthesis grade) and acetonitrile (HPLC grade) were purchased from Biolab Chemicals. Diisopropylethylamine (DIPEA), piperidine, triisopropylsilane (TIS), D₂O and Trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, Israel. All the standard Fmoc-amino acids, Fmoc-Ser(HPO₃Bzl)-OH and Fmoc-Thr(HPO₃Bzl)-OH were purchased from Luxembourg Industries Limited, Israel. Rink amide MBHA polystyrene resin was purchased from GL Bio with 0.546 mmol/g loading.

2. Methods

2.1 General procedure for solid phase peptide synthesis

Solid phase peptide synthesis was performed using a CEM-Discover Microwave assisted Peptide Synthesizer (CEM Corporation, Mathews, NC) using Fmoc chemistry. The maximum temperature for couplings was set at 75 °C with 25 W. All the Phosphorylated peptides were synthesized typically on 0.1 mmol scale.

2.1.1 Coupling procedure for MW-SPPS method

Fmoc-protected amino acid (5 equiv), 4.5 equiv HATU and DIPEA (8 equiv) were dissolved in DMF (6 mL). The mixture was allowed to activate for 5 min at 0 °C and then added to the resin bearing the free amine. The reaction mixture was then microwave-irradiated for 5 min at 75 °C. The resin was allowed to cool to room temperature and was then washed thoroughly with DMF. All the Fmoc deprotections were performed by treating the peptidyl-resin twice with 20% piperidine in DMF for 2 min and 4 min at 75 °C temperature, both using MW irradiation. The solid support was then washed thoroughly with DMF.

Coupling procedure for pSPPS method

Fmoc-protected amino acid (3equiv), 2.5 equiv HATU and DIPEA (6 equiv) were dissolved in DMF (3 mL). The mixture was allowed to activate for 5 min at 0 °C and then added to the resin bound peptide. The solid support was then microwave irradiated for 5 min at 75 °C. The resin was allowed to cool to room temperature and washed thoroughly with DMF and DCM. All the Fmoc deprotections were performed by treating the peptidyl-resin twice with 20% piperidine in DMF for 10 min and 15 min at

room temperature without the use of MW. The solid support was then washed thoroughly with DMF and DCM. All the non-phosphorylated amino acids in this method were coupled using 5 equiv of amino acids, 4.5 equiv of HATU and 8 equiv of DIPEA in 6 mL of DMF employing same conditions as phosphorylated amino acids.

Coupling procedure for ET-pSPPS method

Fmoc-protected amino acid (3 equiv), 2.5 equiv HATU and DIPEA (6 equiv) were dissolved in DMF (3 mL). The mixture was allowed to activate for 5 min at 0 °C and added to the resin bearing the free amine. The solid support was then microwave-irradiated for 10 min at 75 °C. The resin was allowed to cool to room temperature and washed thoroughly with DMF and DCM. All the Fmoc deprotections were performed by treating the peptidyl resin twice with 20% piperidine in DMF for 10 min and 15 min at room temperature without the use of MW. The solid support was then washed thoroughly with DMF and DCM. All the non-phosphorylated amino acids were coupled using 5 equiv of amino acids, 4.5 equiv of HATU and 8 equiv of DIPEA in 6 mL of DMF employing the above conditions.

Coupling procedure for DC-pSPPS method

Fmoc-protected amino acid (3 equiv), 2.5 equiv HATU and DIPEA (6 equiv) were dissolved in DMF (3 mL). The mixture was activated at 0 °C for 5 min. The activated amino acid solution was then added to the resin bound peptide. The double coupling was performed at 75 °C for 5 min each time using 3 equiv of fresh phosphorylated amino acid in each cycle. The resin was washed thoroughly with DMF, DCM and the completion of coupling was confirmed by Kaiser test and HPLC-MS analysis (small cleavage). All the Fmoc deprotections were performed by treating the peptidyl-resin twice with 20% piperidine in DMF for 10 min and 15 min at room temperature without the use of MW. The solid support was then washed thoroughly with DMF and DCM. All the non-phosphorylated amino acids were coupled using 5 equiv of amino acids, 4.5 equiv of HATU and 8 equiv of DIPEA in 6 mL of DMF employing the above conditions.

Coupling procedure for EBB-pSPPS method

Fmoc-protected amino acid (5 equiv), 4.5equiv HATU and DIPEA (8 equiv) were dissolved in DMF (6 ml).The mixture was allowed to activate for 5 min at 0 °C. The activated amino acid solution was then added to the resin bearing the free amine. The 10 min coupling was performed in two cycles using 5 equiv. of fresh phosphorylated amino acid in each cycle under microwave irradiation at 75 °C. The resin was allowed to cool to room temperature and then washed thoroughly with DMF and DCM. The reaction was monitored by Kaiser test. All the Fmoc deprotections were performed by treating the peptidyl resin twice with 20% piperidine in DMF for 10 min and 15 min at room temperature without the use of MW. The solid support was then washed thoroughly with DMF and DCM. All the non-phosphorylated amino acid residues were also incorporated employing the same conditions.

2.1.2 General procedure for conventional Fmoc deprotection

The Fmoc-peptidyl-resin was treated twice with 20% piperidine in DMF (6 mL) for 10 min and 15 min at room temperature. The solid support was then washed thoroughly with DMF (6 X 3 mL) and DCM (3 X 3 mL).

2.1.3 General procedure for microwave Fmoc deprotection

The resin bound Fmoc-peptide was treated twice with 20% piperidine in DMF (6 mL) for 2 min and 4 min at 75 °C under microwave irradiation. The resulting deprotected peptide was allowed to cool to room temperature and thoroughly washed with DMF and DCM.

2.1.4 Procedure for peptide cleavage from the resin

A freshly prepared solution (5 mL) of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/ TDW/ethane dithiol (EDT) (94:1:2.5:2.5) was cooled to 0°C and added to 200 mg resin-bound peptide. The mixture was shaken at room temperature according to the times given below for each sequence. Then, the resin was separated by filtration. The TFA was removed under nitrogen atmosphere and the peptide was precipitated by gradual addition of ice-cold ether to the mixture. The solution was centrifuged and the peptide washed twice with ether. A minimum volume of a 1:2 ACN/TDW mixture was used to dissolve the crude peptide, which was then lyophilized before HPLC purification and MS analysis.

2.2 RP-HPLC analysis

The crude phosphorylated peptides were analyzed by Merck Hitachi HPLC with a reverse-phase Agilent analytical column (eclipse XDB-Agilent C18, 4.6 x 150mm; 5 μ m) using a linear gradient of 1-30% Acetonitrile in water over 30 min with 0.1% TFA.

2.3 RP-HPLC purification

The crude peptides were purified by Merck Hitachi HPLC with a reverse-phase C18 semi prep column (Merck puospher STAR Rp-18e; 5 μ m) with flow rate of 4.5 mL/min using a liner gradient of 2–40 % acetonitrile in water, over 40 min with 0.1% TFA.

2.4 RP-UPLC analysis

Pure phosphorylated peptides were characterized by analytical reversed-phase Acquity UPLC H-Class with the UV detection (220 nm and 280 nm) using a WatersTMXSelect C18 column (3.5 μ m, 130Å, 4.6 x 150mm). The flow rate was set to 1 mL/min using a linear gradient of 1-30% of acetonitrile in water, 0.1% TFA in 30 minutes.

2.5 NMR Analysis

³¹P NMR spectra of all the phosphopeptides were recorded in D₂O using BBO-5mm probe on Bruker advance-II 202.4 MHz instrument.

2.6 Mass spectrometry

Phosphorylated peptides were characterized by Electrospray ionization MS on LCQ Fleet Ion Trap mass spectrometer instrument (Thermo Scientific). Peptides masses were calculated from the experimental mass to charge (m/z) ratios from all of the observed multiply charged species of a peptide. Deconvolution of the experimental MS data was performed with the help of the MagTran v1.03 software.

3. Synthesis of peptide **2** using state of the art

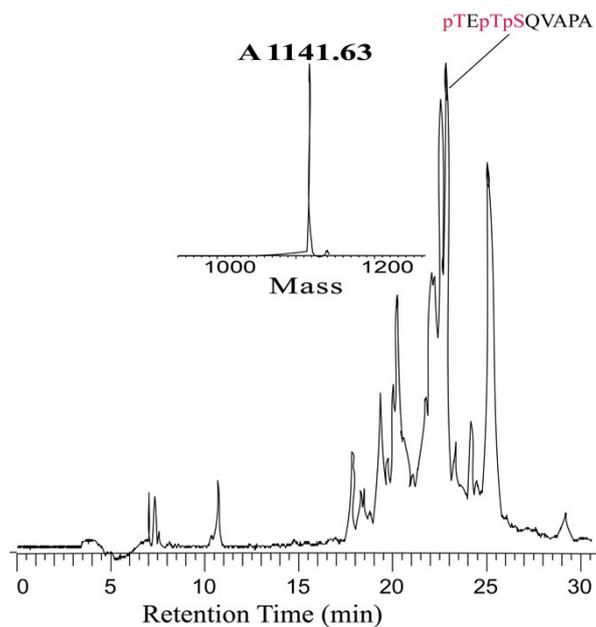


Figure S1. RP-HPLC chromatogram of the peptide (pTEpTpSQVAPA) synthesized employing standard MW assisted phosphopeptide coupling method.

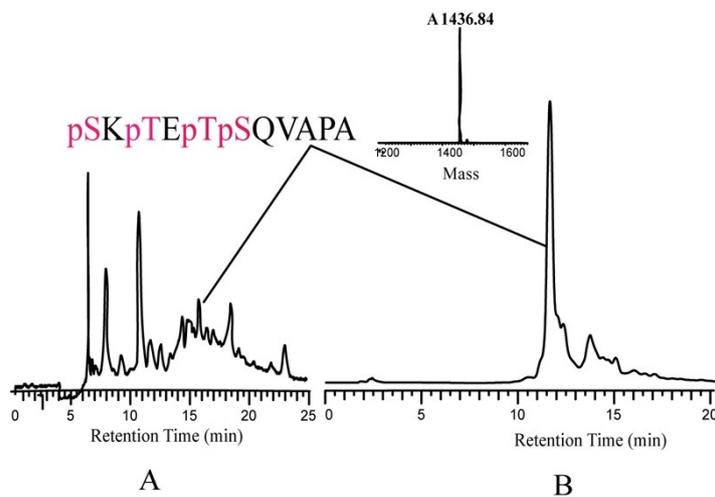


Figure S2. (A) RP-HPLC chromatogram of the peptide (pSKpTEpTpSQVAPA) **7** synthesized employing standard MW assisted phosphopeptide coupling method; (B) Chromatogram of the peptide **7** synthesized using our new combinatorial approach.

Table S1. Coupling methods and conditions used for the synthesis of the Rhodopsin derived seven phosphorylated peptide **1**

method	coupling			Deprotection	
MW-SPPS	Time	5	Time	2 + 4	
	Temp	75°C	Temp	75 °C	
	Cycles	1	MW	Yes	
	Equiv	5			
pSPPS	Time	5	Time	10 + 15	
	Temp	75°C	Temp	rt	
	Cycles	1	MW	No	
	Equiv	3			
ET-pSPPS	Time	10	Time	10 + 15	
	Temp	75°C	Temp	rt	
	Cycles	1	MW	No	
	Equiv	3			
DC-pSPPS	Time	10	Time	10 + 15	
	Temp	75°C	Temp	rt	
	Cycles	2	MW	No	
	Equiv	3			
EBB-pSPPS	Time	10	Time	10 + 15	
	Temp	75°C	Temp	rt	
	Cycles	2	MW	No	
	Equiv	5			

* All the non-phosphorylated amino acids were coupled using five equivalents. ** The equivalents listed in Table S1 are applicable only to phosphorylated amino acids. *** Temperature (°C), Time (min).

4.2 Summary of peptides MS, ³¹P characterization and HPLC purity analysis

Peptide	Sequence	Observed mass (Da)	Calculated mass (Da)	Crude purity	HPLC	No. of p-residues determined by ³¹ P-NMR
1	CDDEApSpTpTVpSKpTEpTpSQVAPA	2598.46	2597.90	78		7
2	DDEApSpTpTVpSKpTEpTpSQVAPA	2496.22	2495.66	80		7
3	CDDEASpTpTVpSKpTEpTpSQVAPA	2518.56	2517.90	79		6
4	pTpTVpSKpTEpTpSQVAPA	1897.70	1898.52	80		6
5	ASpTpTVpSKpTEpTSQVAPA	1976.35	1976.62	87		5
6	CDDEApSpTpTVpSKpTETSQVAPA	2438.65	2437.90	82		5
7	pSKpTEpTpSQVAPA	1436.84	1435.36	88		4
8	DDEApSTpTVSKpTETpSQVAPA	2255.63	2255.76	90		4
9	DDEASpTpTVpSKTETSQVAPA	2174.87	2175.79	87		3
10	CDDEASpTpTVSKTETpSQVAPA	2278.44	2279.90	91		3
11	DDEASTVpSKTETSQVA	2015.51	2015.86	92		1

Table S2. The library of phosphorylated peptides with different phosphorylation patterns synthesized in this study

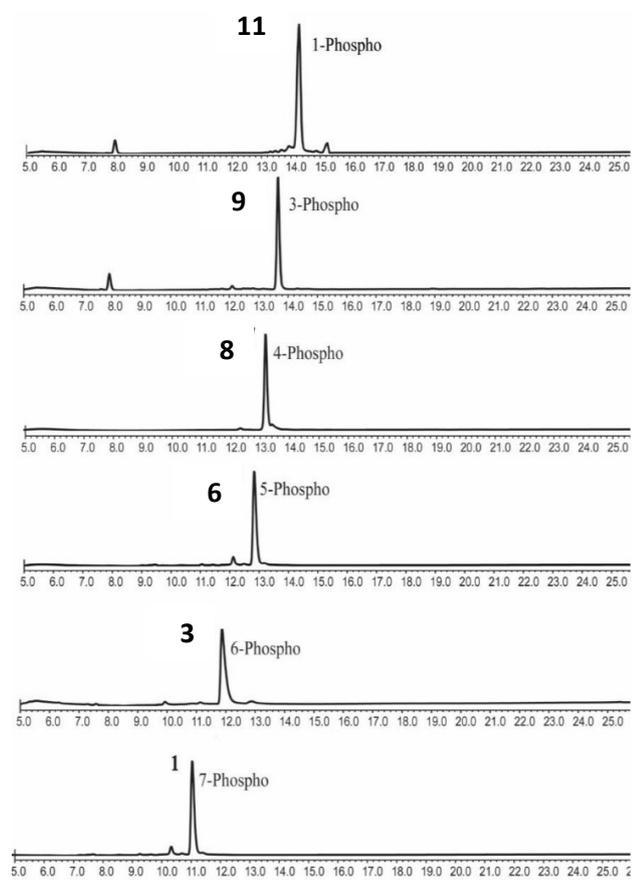


Figure S3. Overlay of UPLC profiles of the synthesized multi-phosphorylated peptides with the increasing number of phosphorylation sites.

5. Detailed synthetic protocols and analysis of the peptide library

Synthesis and characterization of peptide 1

Peptide 1 was synthesized following the sequence of coupling methods described in Fig. S4. The peptide was fully cleaved from the solid support following 8 hours incubation in the TFA mixture as described above.

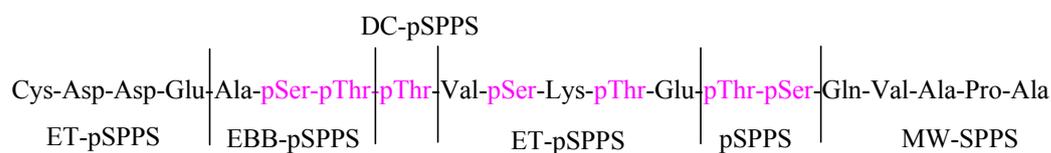


Figure S4. The sequence of coupling methods used in the synthesis of peptide 1.

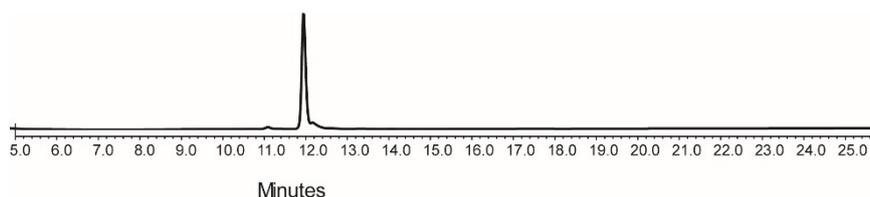


Figure S5. RP-UPLC Chromatogram of peptide 1 (CDDEApSpTpTVpSKpTEpTpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-Class XSelect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220nm).

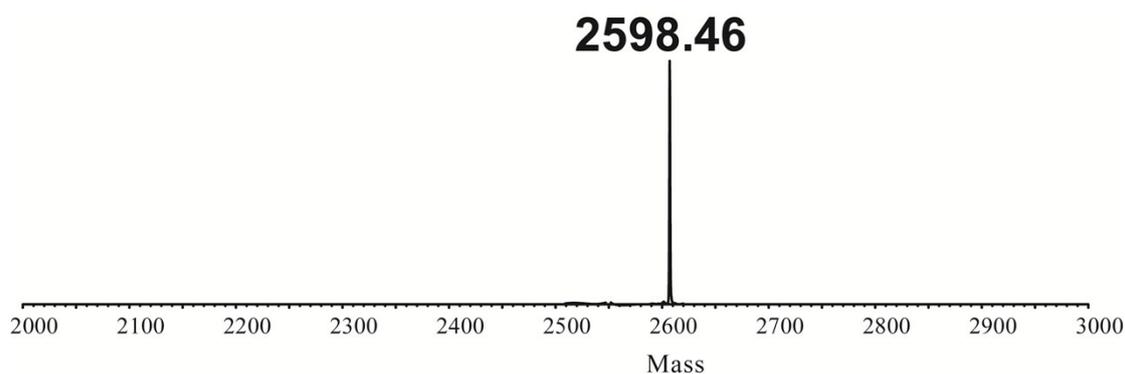


Figure S6. Deconvoluted mass spectra of peptide 1 (CDDEApSpTpTVpSKpTEpTpSQVAPA).

Obs. 2598.46 Da; Calc. 2599.02 Da.

Synthesis and characterization of peptide 2

Peptide 3 was synthesized following the sequence of coupling methods described in Fig. S10. The peptide was fully cleaved from the solid support following 8 hours incubation in the TFA mixture as described above.

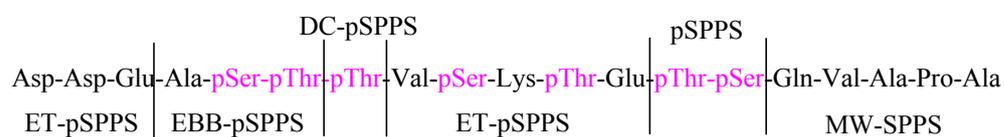


Figure S7. The sequence of methods used in the synthesis of peptide 2.

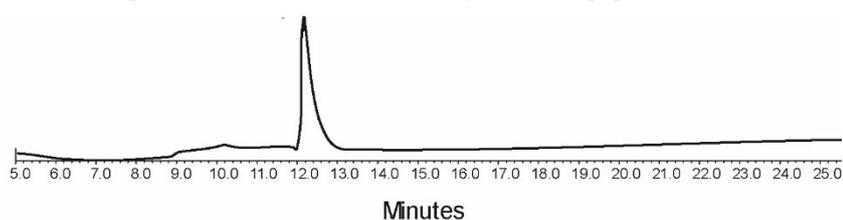


Figure S8. RP-UPLC Chromatogram of peptide 2 (DDEApSpTpTVpSKpTEpTpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6 x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

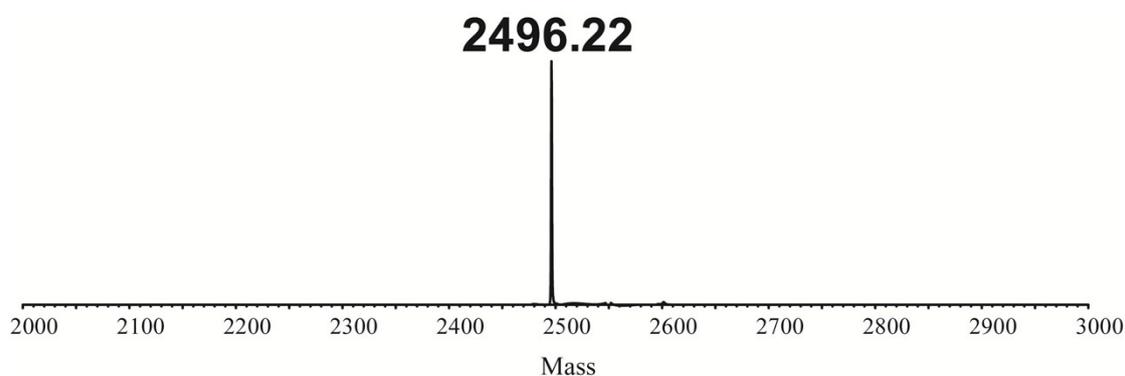


Figure S9. De convoluted mass spectra of peptide 2 (DDEApSpTpTVpSKpTEpTpSQVAPA).

Obs. 2496.22 Da; **Calc.** 2495.87 Da.

Synthesis and characterization of peptide 3

Peptide 3 was synthesized following the sequence of coupling methods described in Fig. S31. The peptide was fully cleaved from the solid support following 7.5 hours incubation in the TFA mixture as described above.

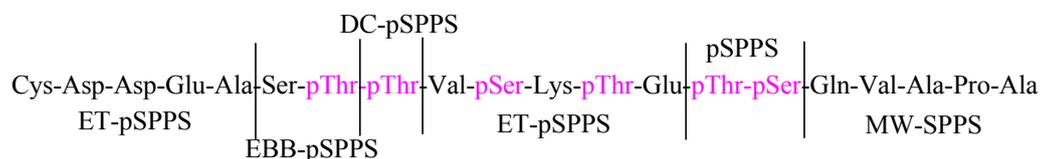


Figure S10. The sequence of coupling methods used in the synthesis of peptide 3.

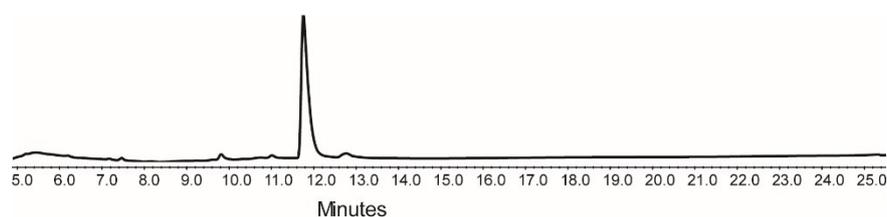


Figure S11. RP-UPLC Chromatogram of peptide 3 (CDDEASpTpTVpSKpTEpTpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

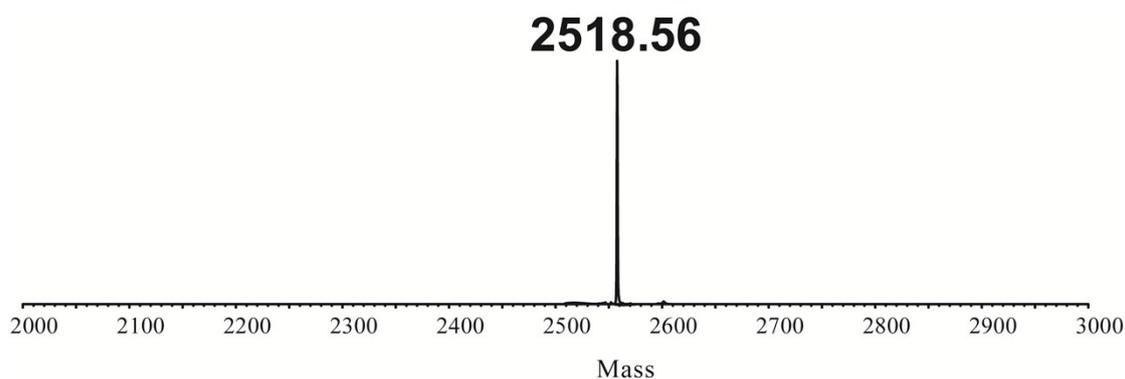


Figure S12. De convoluted mass spectra of peptide 3 (CDDEASpTpTVpSKpTEpTpSQVAPA).

Obs. 2518.56 Da; **Calc.** 2519.03 Da.

Synthesis and characterization of peptide 4

Peptide 4 was synthesized following the sequence of coupling methods described in Fig. S34. The peptide was fully cleaved from the solid support following 7 hours incubation in the TFA mixture as described above.

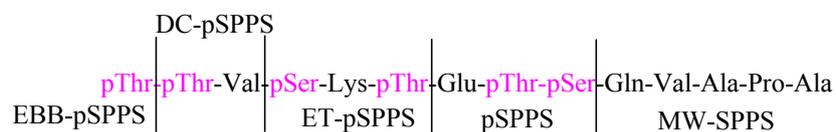


Figure S13. The sequence of coupling methods used in the synthesis of peptide 4.

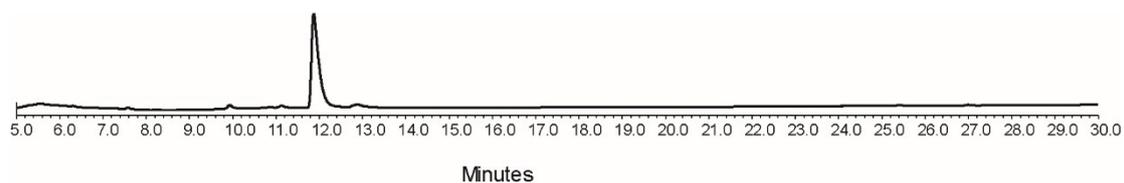


Figure S14. RP-UPLC Chromatogram of peptide 4 (pTpTVpSKpTEpTpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

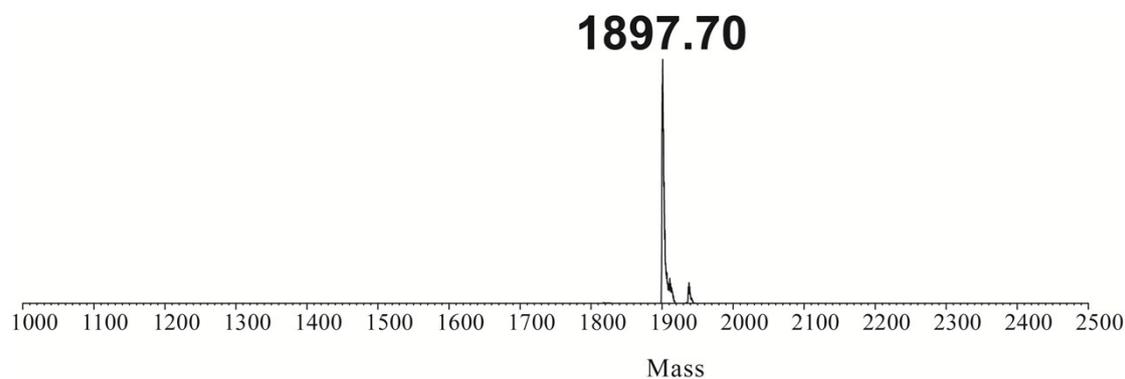


Figure S15. De convoluted mass spectra of peptide 4 (pTpTVpSKpTEpTpSQVAPA).

Obs. 1897.70 Da; **Calc.** 1898.44 Da.

Synthesis and characterization of peptide 5

Peptide 5 was synthesized following the sequence of coupling methods described in Fig. S25. The peptide was fully cleaved from the solid support following 6 hours incubation in the TFA mixture as described above.

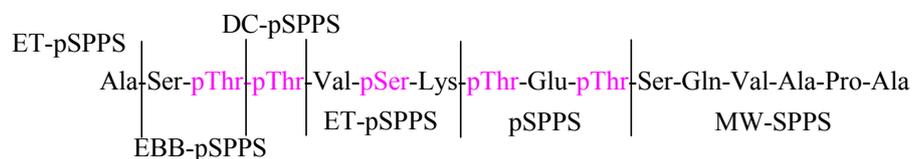


Figure S16. The sequence of coupling methods used in the synthesis of peptide 5.

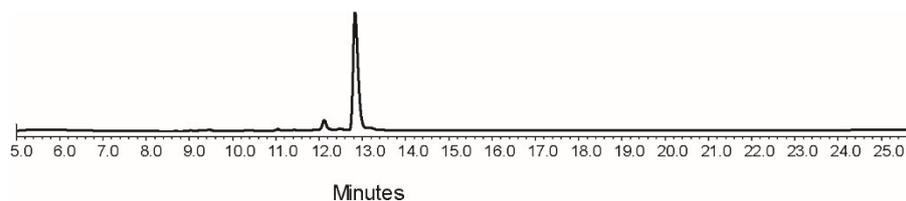


Figure S17. RP-UPLC Chromatogram of peptide 5 (ASpTpTVpSKpTEpTSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

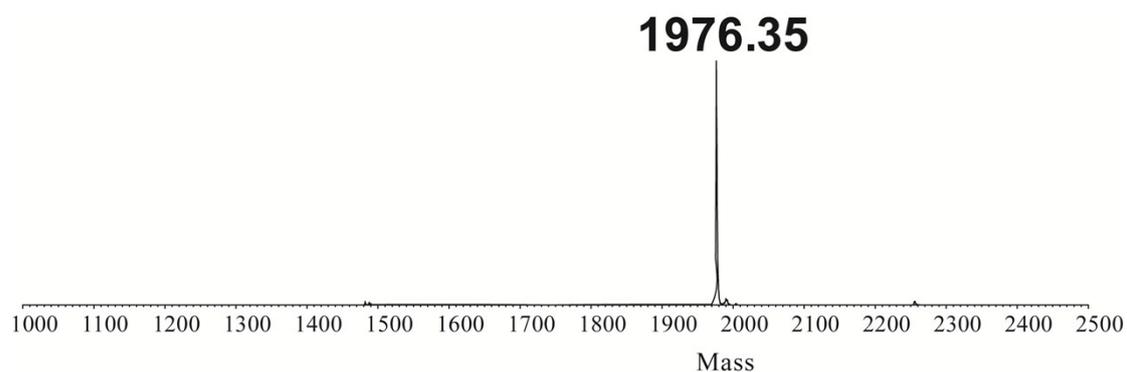


Figure S18. De convoluted mass spectra of peptide 5 (ASpTpTVpSKpTEpTSQVAPA).

Obs.1976.35 Da; **Calc.**1976.62 Da.

Synthesis and characterization of peptide 6

Peptide 6 was synthesized following the sequence of coupling methods described in Fig. S28. The peptide was fully cleaved from the solid support following 6.5 hours incubation in the TFA mixture as described above.

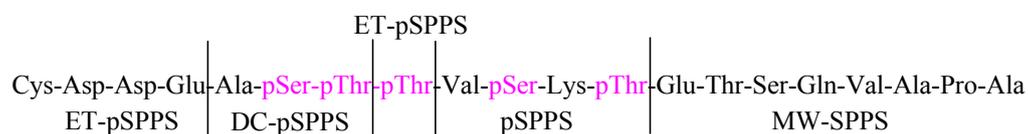


Figure S19. The sequence of coupling methods used in the synthesis of peptide 6.

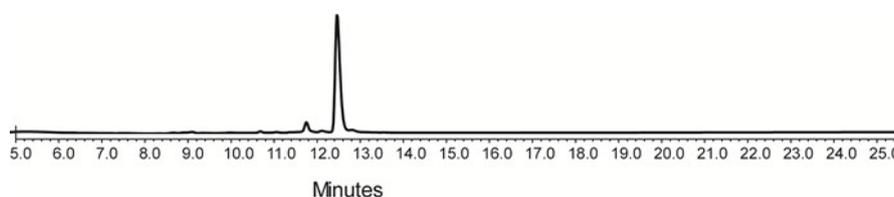


Figure S20. RP-UPLC Chromatogram of peptide 6 (CDDEApSpTpTVpSKpTETSQVAPA).
(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

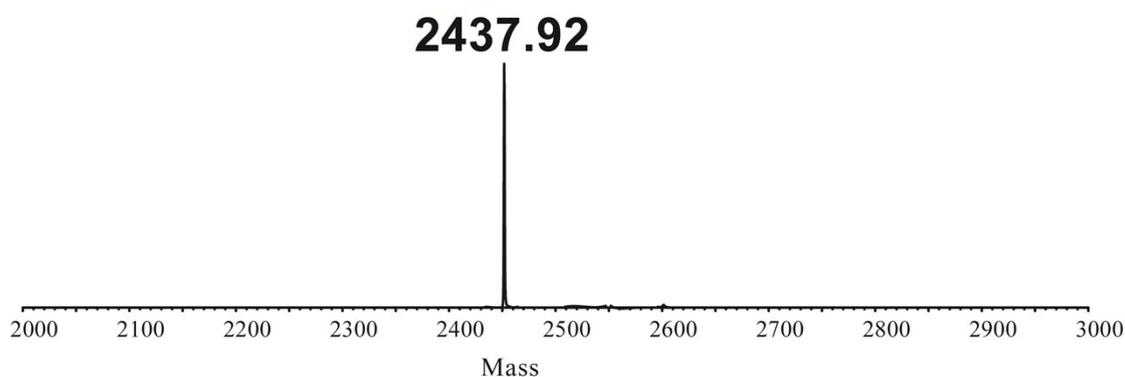


Figure S21. De convoluted mass spectra of peptide 6 (CDDEApSpTpTVpSKpTETSQVAPA).

Obs. 2437.92 Da; **Calc.** 2439.05Da.

Synthesis and characterization of peptide 7

Peptide 7 was synthesized following the sequence of coupling methods described in Fig. S7. The peptide was fully cleaved from the solid support following 5 hours incubation in the TFA mixture as described above.

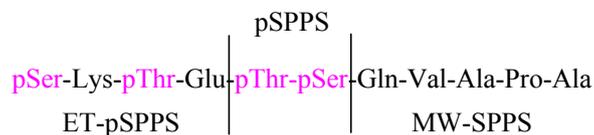


Figure S22. The sequence of methods used in the synthesis of peptide 7.

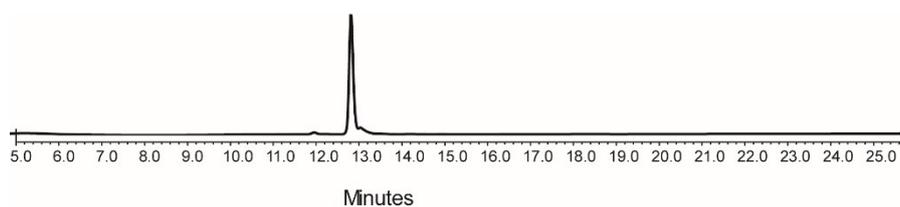


Figure S23. RP-UPLC Chromatogram of peptide 7 (pSKpTEpTpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

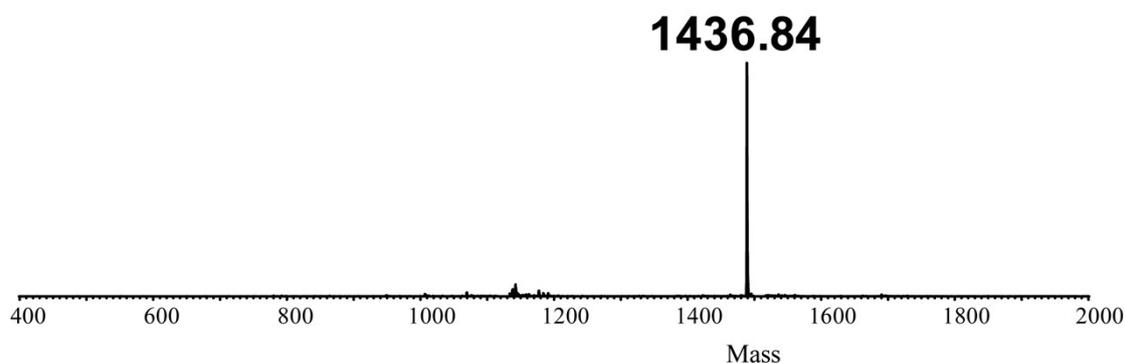


Figure S24. De convoluted mass spectra of peptide 7 (pSKpTEpTpSQVAPA).

Obs. 1436.84 Da; **Calc.** 1437.13 Da.

Synthesis and characterization of peptide 8

Peptide **8** was synthesized following the sequence of coupling methods described in Fig. S22. The peptide was fully cleaved from the solid support following 5.5 hours incubation in the TFA mixture as described above.

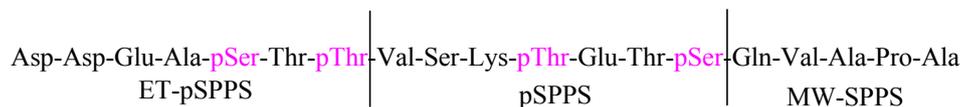


Figure S25. The sequence of coupling methods used in the synthesis of peptide **8**.

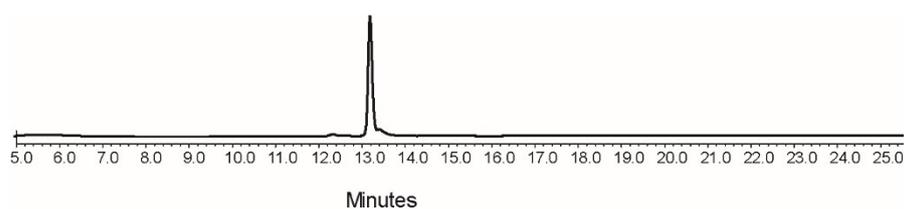


Figure S26. RP-UPLC Chromatogram of peptide **8** (DDEApSTpTVSKpTETpSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

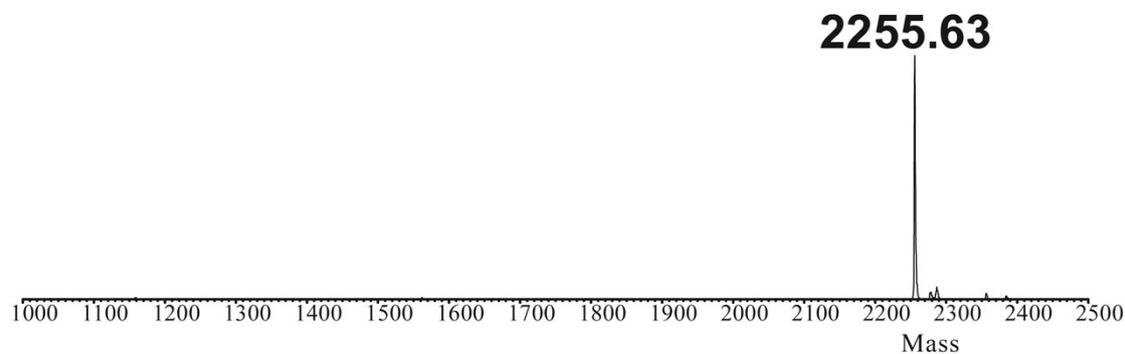


Figure S27. De convoluted mass spectra of peptide **8** (DDEApSTpTVSKpTETpSQVAPA).

Obs. 2255.63 Da; **Calc.** 2255.92 Da.

Synthesis and characterization of peptide 9

Peptide **9** was synthesized following the sequence of coupling methods described in Fig. S16. The peptide was fully cleaved from the solid support following 4.5 hours incubation in the TFA mixture as described above.

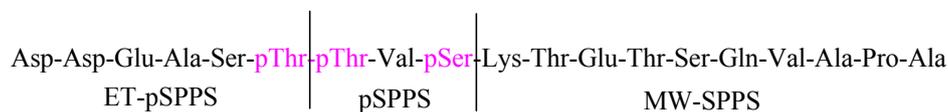


Figure S28. The sequence of coupling methods used in the synthesis of peptide **9**.

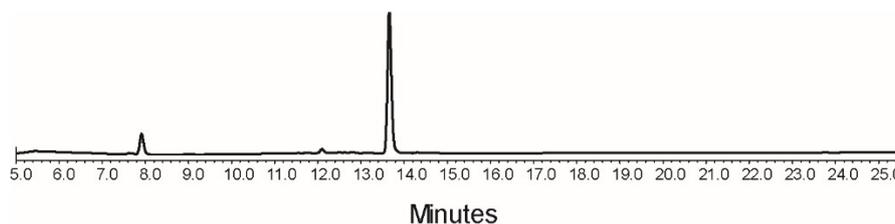


Figure S29. RP-UPLC Chromatogram of peptide **9** (DDEASpTpTVpSKTETSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

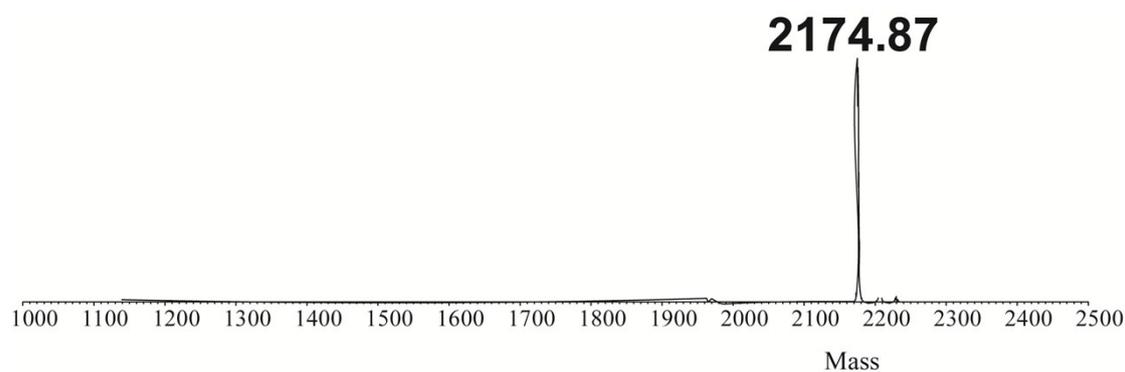


Figure S30. De convoluted mass spectra of peptide **9** (DDEASpTpTVpSKTETSQVAPA).

Obs. 2174.87 Da; **Calc.** 2175.94 Da.

Synthesis and characterization of peptide 10

Peptide **10** was synthesized following the sequence of coupling methods described in Fig. S19. The peptide was fully cleaved from the solid support following 4.5 hours incubation in the TFA mixture as described above.

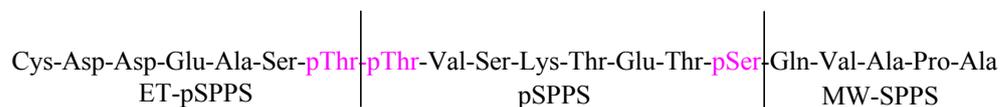


Figure S31. The sequence of coupling methods used in the synthesis of peptide **10**.

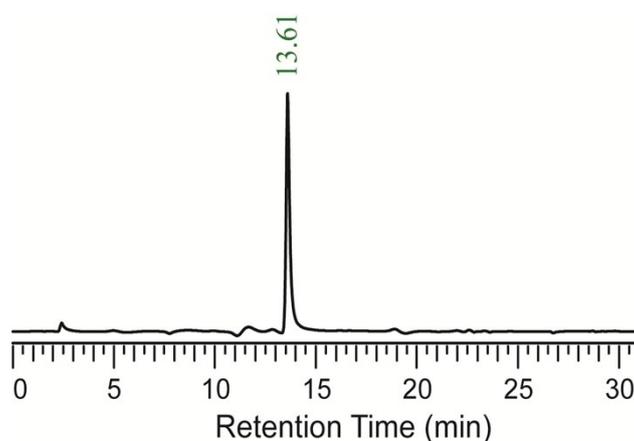


Figure S32. RP-HPLC Chromatogram of peptide **10** (CDDEASpTpTVSKTETpSQVAPA).

(**conditions:** flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

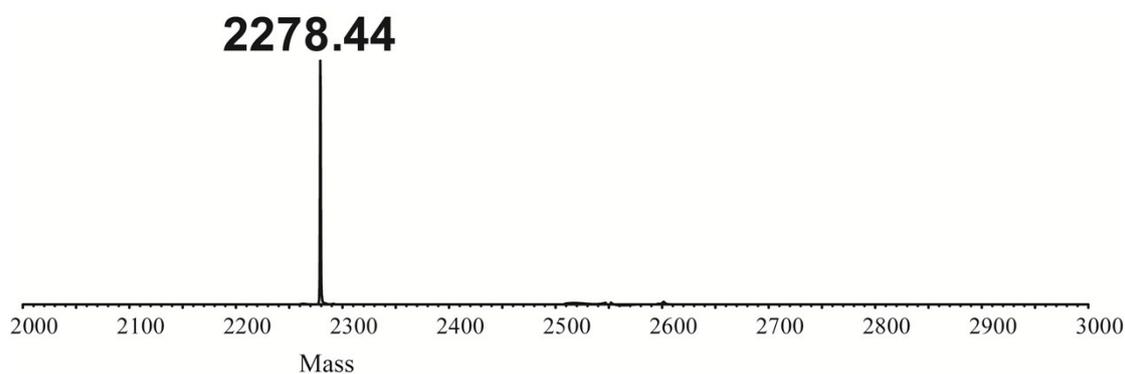


Figure S33. De convoluted mass spectra of peptide **10** (CDDEASpTpTVSKTETpSQVAPA).

Obs. 2278.44 Da; **Calc.** 2279.08 Da.

Synthesis and characterization of peptide 11

Peptide **11** was synthesized following the sequence of coupling methods described in Fig. S13. The peptide was fully cleaved from the solid support following 3 hours incubation in the TFA mixture as described above.



Figure S34. The sequence of coupling methods used in the synthesis of peptide **11**.

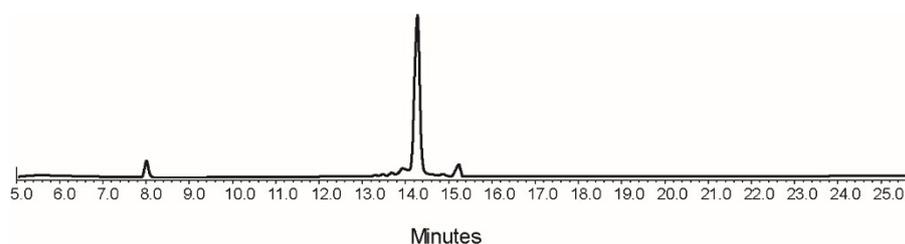


Figure S35. RP-UPLC Chromatogram of peptide **11** (DDEASTTVpSKTETSQVAPA).

(conditions: flow rate: 1 mL/min; Acquity UPLC H-class Xselect C18 column, 4.6x 150 mm; 3.5 μ m; gradient: ACN: H₂O 1-30 % ACN in 30 min; 220 nm).

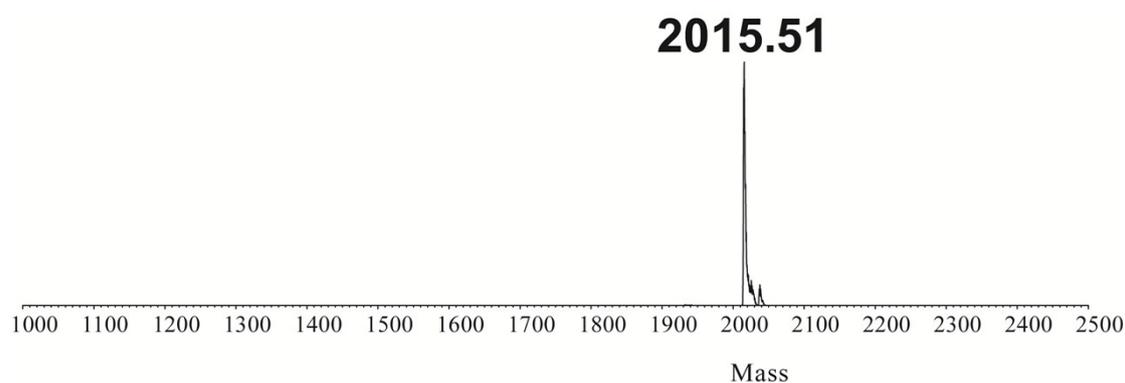


Figure S36. De convoluted mass spectra of peptide **11** (DDEASTTVpSKTETSQVAPA).

Obs.2015.51 Da; **Calc.**2015.98 Da.

6. Detailed ^{31}P NMR spectra of multi-phosphorylated peptides

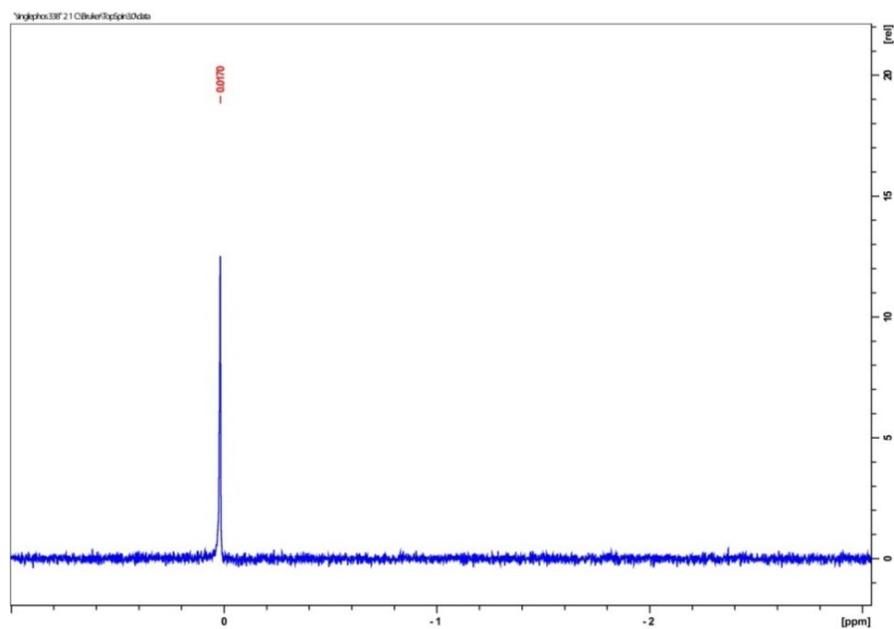


Figure S37. ^{31}P NMR spectra of peptide 11 (DDEASTTV_pSKTETSQVAPA).

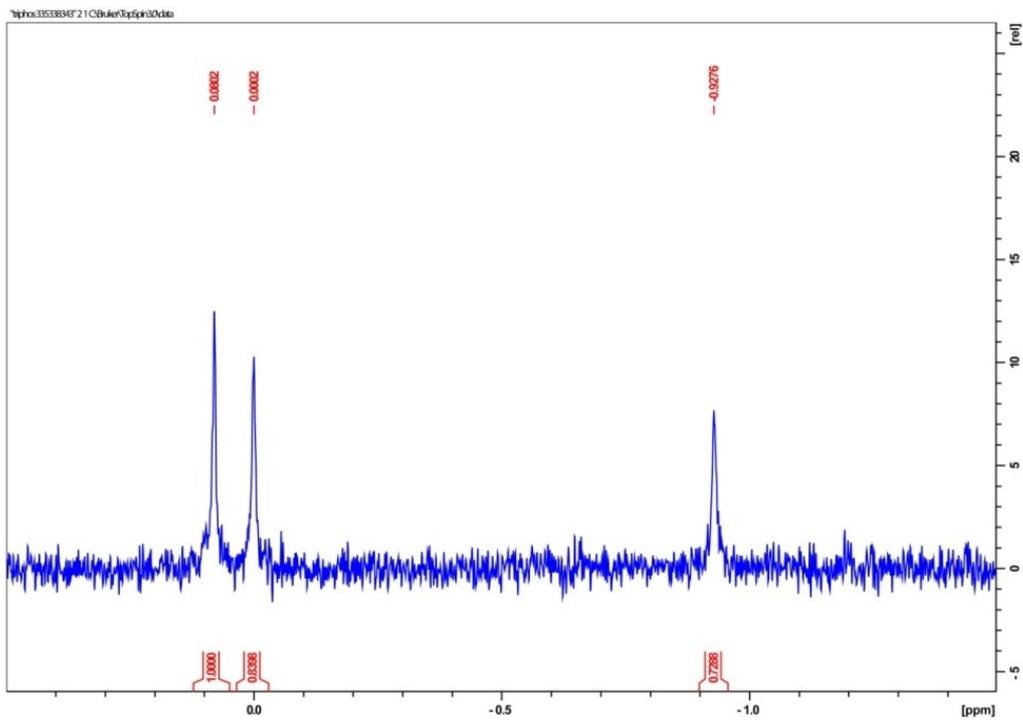


Figure S38. ³¹P NMR spectra of peptide 9 (DDEASpTpTVpSKTETSQVAPA).

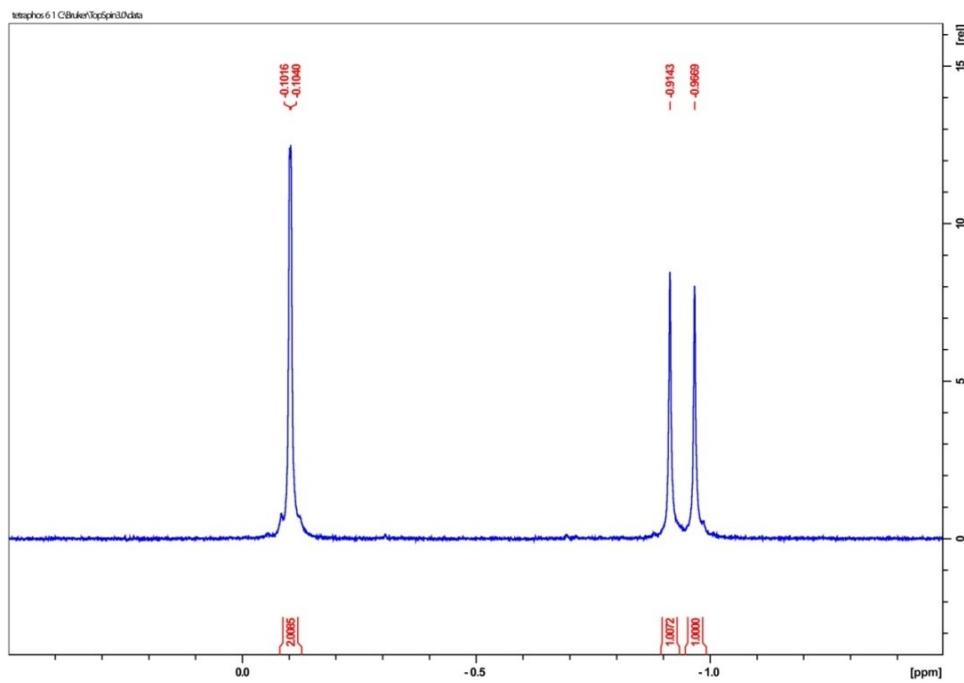


Figure S39. ^{31}P NMR spectra of peptide 8 (DDEApSTpTVSKpTETpSQVAPA).

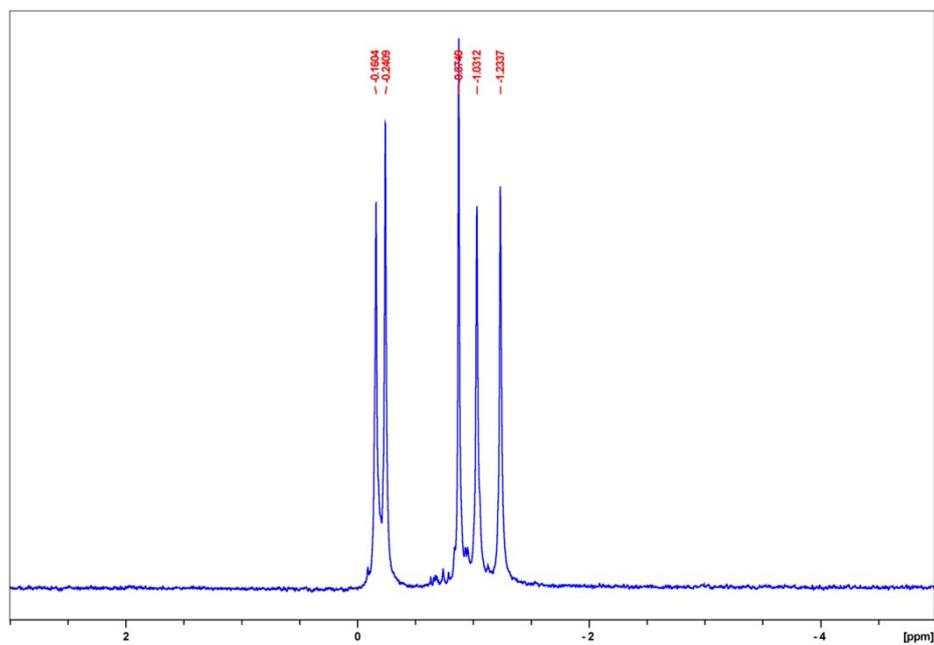


Figure S40. ^{31}P NMR spectra of peptide 6 (CDDEApSpTpTVpSKpTETSQVAPA).

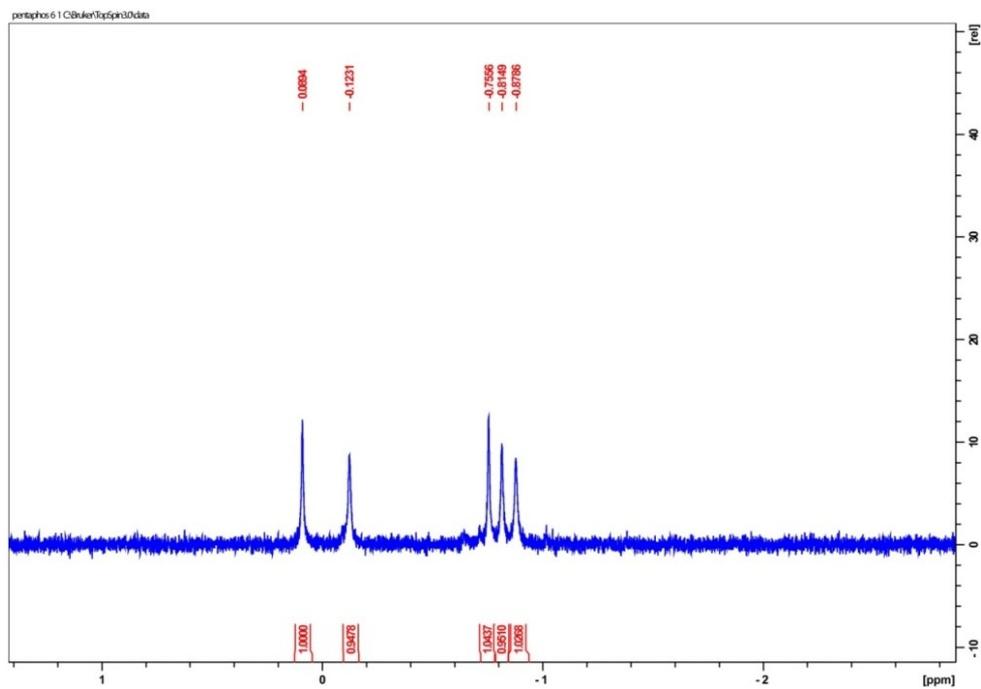


Figure S41. ^{31}P NMR spectra of peptide **5** (ASpTpTVpSKpTEpTSQVAPA).

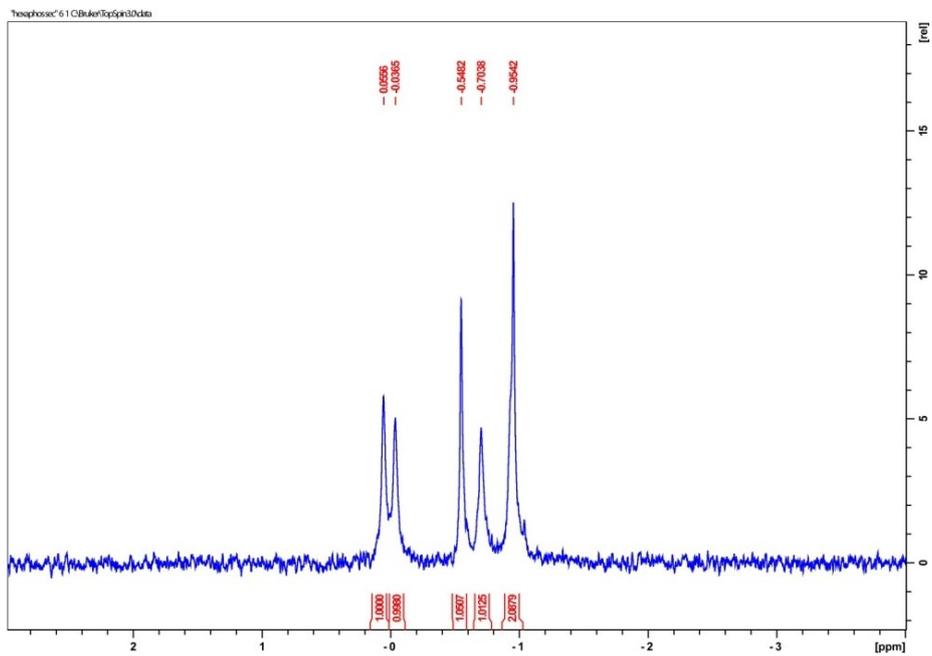


Figure S42. ^{31}P NMR spectra of peptide **3** (CDDEASpTpTVpSKpTEpTpSQVAPA).

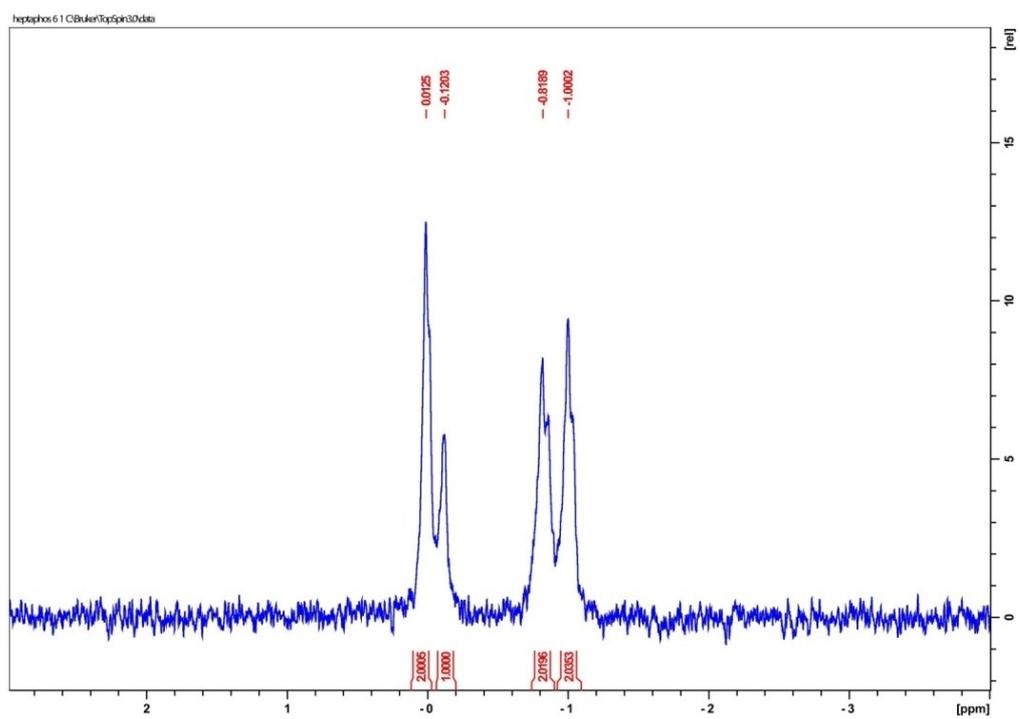


Figure S43. ^{31}P NMR spectra of peptide **1** (CDDEApSpTpTVpSKpTEpTpSQVAPA).