Supplementary Material (ESI) for Chemical Communications

Supporting Information

Phosphate-Assisted Peptide Ligation

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General materials and methods

Analytical reverse-phase HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. A Waters Sunfire 5 μm, 2.1 x 150 mm column was used at a flow rate of 0.2 mL min^{-1} and results analysed with Waters Empower software. Preparative reverse-phase HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 and 280 nm. A Waters Sunfire 5 μm, 19 x 150 mm column was used at a flow rate of 7 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). Semi-preparative HPLC was performed on a Waters 600 Multisolvent Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 and 280 nm. A Grace Vydac "Protein and Peptide C18", 250 x 10 mm, 10-15 μM particle size column was used at a flow rate of 4 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B).

Ligation yields were determined by weighing on a high accuracy balance (Mettler Toledo XS105 Dual Range Balance) with weighing errors of ± 0.03 mg.

LC-MS was performed on a Thermo Separation Products: Spectra System consisting of P400 Pump and a UV6000LP Photodiode array detector on a Phenomenex Jupiter 5 μm, 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} coupled to a Thermoquest Finnigan LCQ Deca mass spectrometer (ESI) operating in positive mode. Separations involved a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B).

ESI mass spectrometry was performed on a Thermoquest Finnigan LCQ Deca mass spectrometer operating in positive mode.

Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem. DCM and methanol were distilled from calcium hydride. DMF was obtained as peptide synthesis grade from Auspep or Labscan.

Synthesis of peptides and phosphopeptides (1-4) via the Fmoc strategy

Solid-phase peptide synthesis was carried out in syringes, equipped with teflon filters, purchased from Torviq.

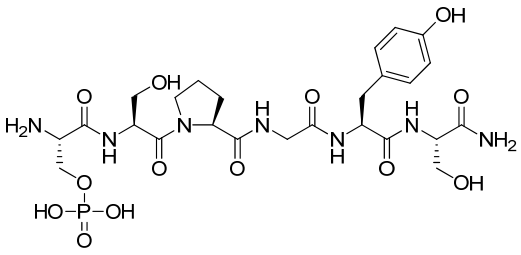
Preloading Rink Amide resin

Rink amide resin was initially washed with DCM (x 5) and DMF (x 5), followed by removal of the Fmoc group by treatment with 10% piperidine/DMF (2 x 5 min). The resin was washed DMF (x 5), DCM (x 5), DMF (x 5). PyBOP (4 eq.) and NMM (8 eq.) was added to a solution of Fmoc-Ser(OtBu)-OH (4 eq.) in DMF (final concentration 0.1 M). After 5 min of pre-activation, the mixture was added to the resin. After 2 h the resin was washed DMF (x 5), DCM (x 5), DMF (x 5), capped with acetic anhydride/pyridine (1:9 v/v) (2 x 5 min) and washed DMF (x 5), DCM (x 5) and DMF (x 5).

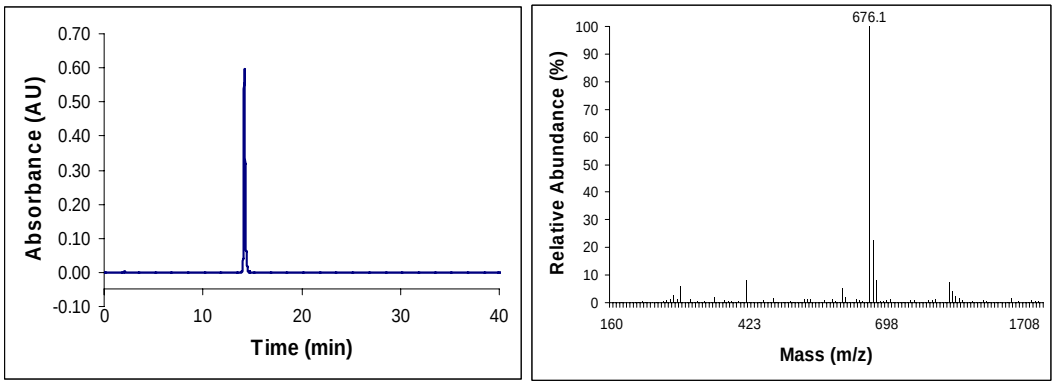
Iterative peptide assembly: *Deprotection:* The resin was treated with 10% piperidine/DMF (2 x 5 min) and washed with DMF (x 5), DCM (x 5) and DMF (x 5). *Amino acid coupling:* A preactivated solution of protected amino acid (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (final concentration 0.1 M) was added to the resin. After 45 min, the resin was washed with DMF (x 5), DCM (x 5) and DMF (x 5). *Capping*: Acetic anhydride/pyridine (1:9 v/v) was added to the resin. After 5 min the resin was washed with DMF (x 5), DCM (x 5) and DMF (x 5). *Cleavage*: A mixture of TFA, thioanisole, triisopropylsilane (TIS) and water (17:1:1:1 v/v/v/v) was added to the resin. After 2 h, the resin was washed with TFA (3 x 2 mL) *Work-up*: The combined solutions were concentrated *in vacuo*. The residue was dissolved in water containing 0.1% TFA and purified by preparative HPLC (gradient 0 to 50% B over 40 min) and analyzed by LC-MS (0 to 50% B over 30 min) and ESI mass spectrometry.

Analytical data for peptides and phosphopeptides 1-4

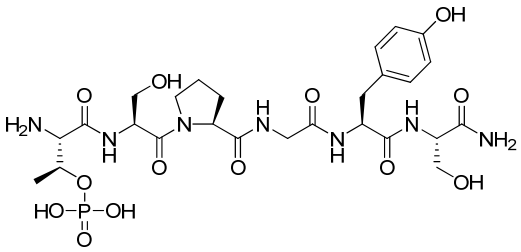
H-pSSPGYS-NH_2 (1)



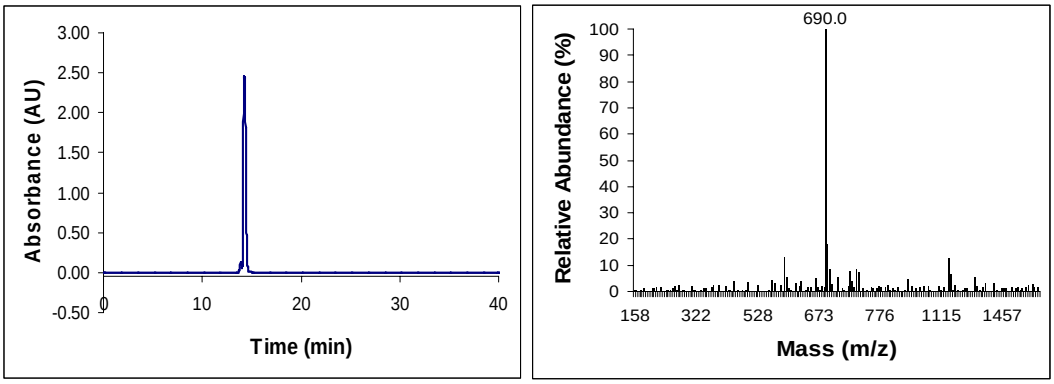
50 μM scale, Yield = 32 mg, 95%; ESI (m/z): Calculated Mass $[M+H]^+$: 676.2, Mass Found 676.1; HPLC: t_R: 14.2 min (Gradient 0 to 50% B over 40 min.).



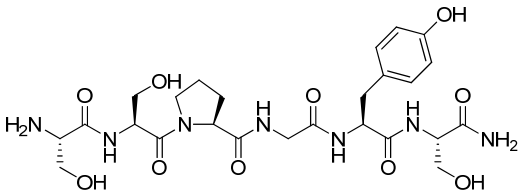
H-pTSPGYS-NH_2 (2)



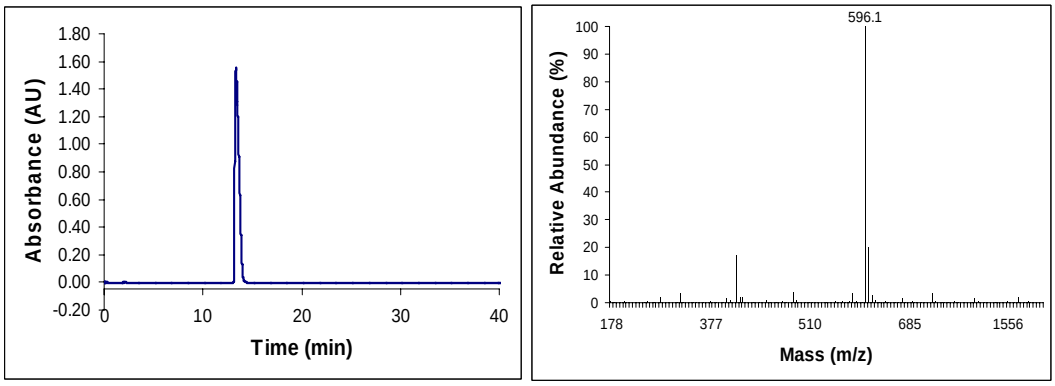
50 μM scale, Yield = 30 mg, 87%; ESI (m/z): Calculated Mass $[M+H]^+$: 690.3, Mass Found 690.0; HPLC: t_R: 14.3 min (Gradient 0 to 50% B over 40 min.).



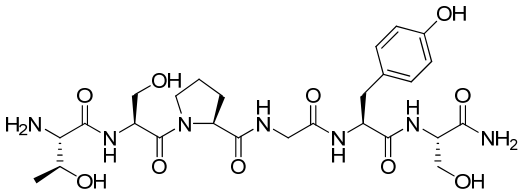
H-SSPGYS-NH_2 (3)



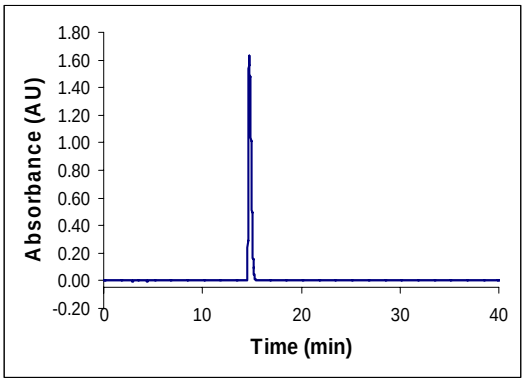
50 μM scale, Yield = 29 mg, 97%; ESI (m/z): Calculated Mass $[M+H]^+$: 596.3, Mass Found 596.1; HPLC: t_R: 13.3 min (Gradient 0 to 50% B over 40 min.).

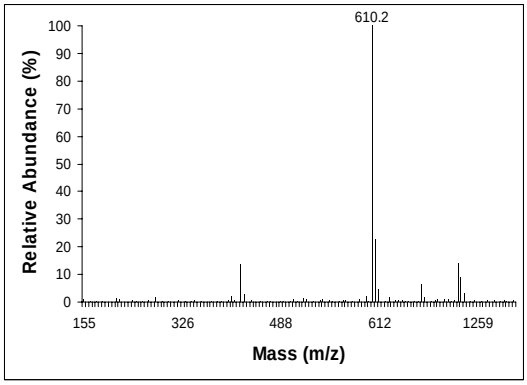


H-TSPGYS-NH_2 (4)



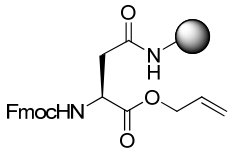
50 μM scale, Yield = 28 mg, 92%; Calculated Mass $[M+H]^+$: 610.3, Mass Found 610.2; HPLC: t_R: 14.7 min (Gradient 0 to 50% B over 40 min.).





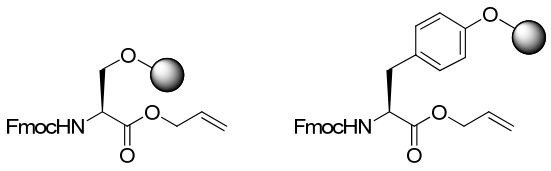
Synthesis of peptide thioesters (5-11) via the side chain anchoring strategy

Side chain anchoring of amino acids onto Rink Amide resin:



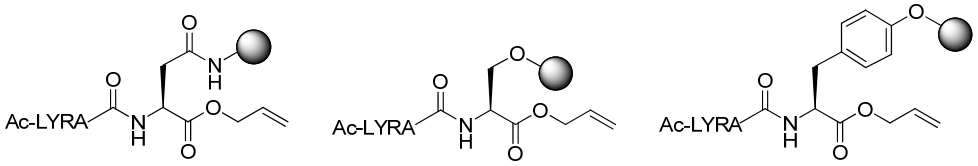
Rink Amide resin co. (100-200 mesh; 1% DVB) (290 mg, loading = 0.69 mmol/g, 200 µmol) was initially washed with DMF (x 5), DCM (x 5) and DMF (x 5), treated with DMF/piperidine (9:1) (2x 5 min) and washed with DMF (x 5), DCM (x 5) and DMF (x 5). A solution of Fmoc-Asp-OAllyl (800 µmol, 4 eq.) in dry DMF (4 ml) containing PyBOP (416 mg, 800 µmol, 4 eq.) and *N*-methylmorpholine (176 µl, 1600 µmol, 8 eq.) was preactivated for 4 min and then added to the resin. After two hours of shaking, the resin was washed with DMF (x 5), DCM (x 5) and DMF (x 5), treated with Ac_2O/Py (1:9 v/v) for 10 min and then washed with DMF (x 5), DCM (x 5) and DMF (x 5). Treatment of the resin with 10% piperidine/DMF (2 x 5 min) and measurement of the resulting fulvene-piperidine adduct at λ = 302 nm showed that the yield of the side chain anchoring was quantitative.

Side chain anchoring onto bromo-(4-methoxyphenyl)methylpolystyrene:



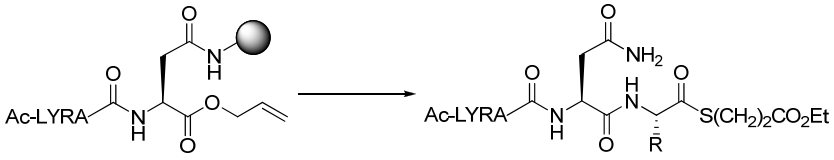
Bromo-(4-methoxyphenyl)methylpolystyrene resin (174 mg, loading = 2.3 mmol/g, 400 μmol) was initially washed with DMF (x 5) and DCM (x 10) and the resin swelled with DCM (5 mL) for 1 h with the exclusion of light. A solution of Fmoc-Ser-OAllyl[1] or Fmoc-Tyr-OAllyl[1] (1200 μmol, 3 eq.), DIEA (2400 μmol, 397 μL, 6 eq.) in DCM (4 mL) was added to the resin, which was shaken for 18-46 h at rt with the exclusion of light. The resin was washed with DCM (x 5) and DMF (x 10). Treatment of the resin with 10% piperidine/DMF (2x 5 min) and measurement of the resulting fulvene-piperidine adduct at $\lambda = 302$ nm was used to determine the loading. NB: The commercially available resin is sold with a very high loading, therefore preloadings of the resins were conducted for a length of time that allowed for the resin to be loaded no more than 1.4 mmol/g to prevent resin crowding and product aggregation. Final loadings: Fmoc-Ser-OAllyl (18 h): loading = 1.28 mmol/g; Fmoc-Tyr-OAllyl (46 h): loading = 1.02 mmol/g;

Solid phase peptide assembly



Peptide assembly was conducted as described for the iterative peptide synthesis above.

General procedure for the *C*-terminal introduction of amino acid thioesters:[1]

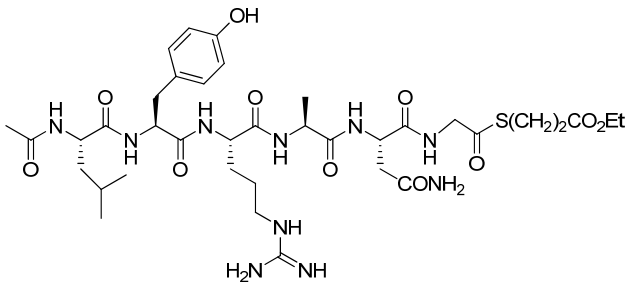


The resin (25 μmol) was swollen in dry DCM for 30 min, followed by the addition of a solution of $Pd(PPh_3)_4$ (25 mg, 22 μmol) and $PhSiH_3$ (123 μl, 108 mg, 1 mmol, 40 eq.) in dry DCM (2 mL). The resin was shaken for 1 h and the procedure was

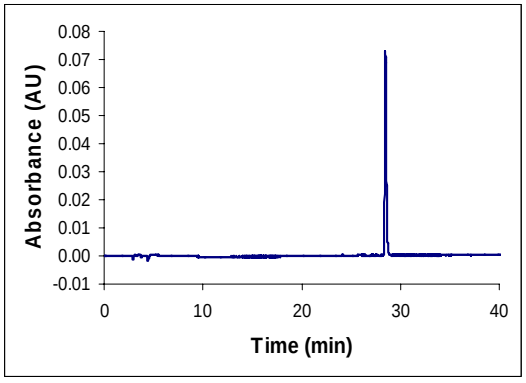
repeated once. Afterwards, the resin was washed with DCM (x 10), DMF (x 5) and DCM (x 5). The amino acid thioester (Gly-SR, Ala-SR, Met-SR, Phe-SR, Ser-SR, Val-SR)[1] (250 µmol, 10 eq.) and DIEA (86 µl, 65 mg, 500 µmol, 20 eq.) were dissolved in dry DCM (1 ml) and added to the resin. HATU (95 mg, 250 µmol, 10 eq.) was added as a solid and the resin was shaken for 1 h. The procedure was repeated once and the resin was washed with DCM (x 5), DMF (x 5) and DCM (x 10). *Cleavage*: A mixture of TFA, thioanisole, triisopropylsilane and water (17:1:1:1 v/v/v/v) was added to the resin. After 2 h, the resin was washed with TFA (4x 4 mL) *Work-up*: The combined solutions were concentrated *in vacuo*. The residue was dissolved in water containing 0.1% TFA and purified by preparative HPLC (0 to 50% B over 40 min.) to afford the desired peptide thioesters as white fluffy solids which were analyzed by HPLC (0 to 50% B over 40 min) and ESI mass spectrometry.

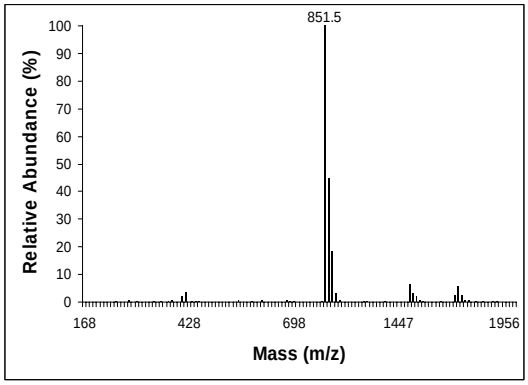
Analytical data for peptide thioesters 5-8 and 11:

Ac-LYRANG-S(CH$_2$)$_2$CO$_2$Et (5)

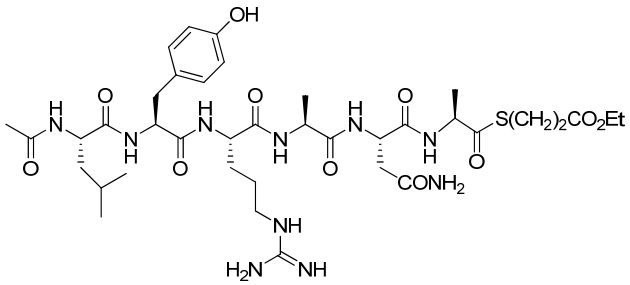


25 µmol of fully assembled Rink amide resin was treated with H-Gly-S(CH$_2$)$_2$CO$_2$Et according to the general procedure described above. Yield: 21.3 mg, quant.; Calculated Mass $[M+H]^+$: 851.4, Mass Found 851.5; HPLC: t_R: 28.5 min (Gradient 0 to 50% B over 40 min.).

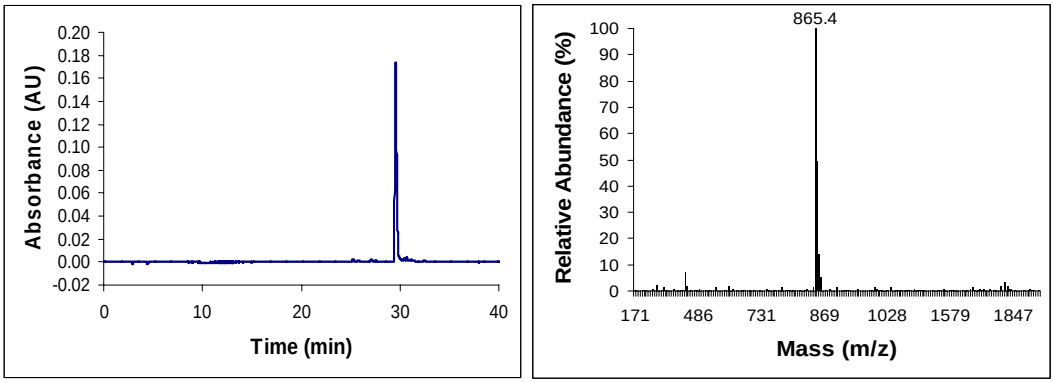




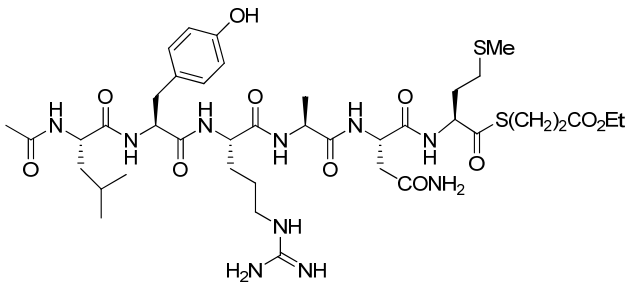
Ac-LYRANA-S(CH$_2$)$_2$CO$_2$Et (6)



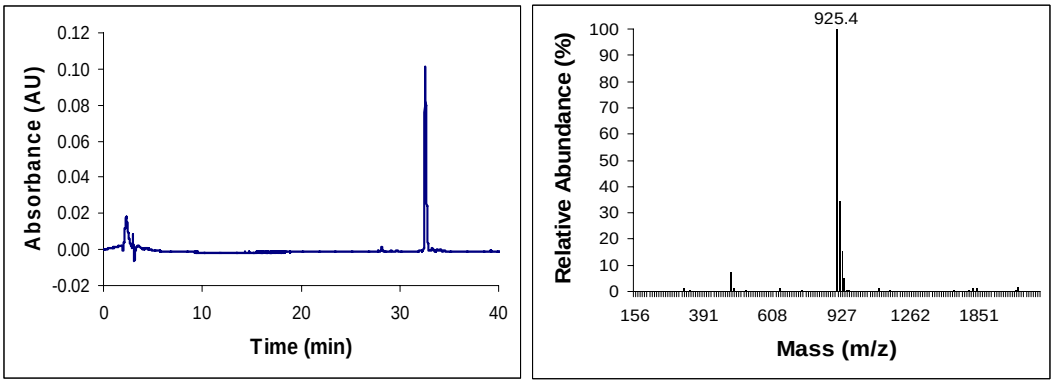
25 µmol of fully assembled Rink amide resin was treated with H-Ala-S(CH$_2$)$_2$CO$_2$Et according to the general procedure described above. Yield: 21.7 mg, quant.; Calculated Mass $[M+H]^+$: 865.4, Mass Found 865.4; HPLC: t_R: 29.5 min (Gradient 0 to 50% B over 40 min.).



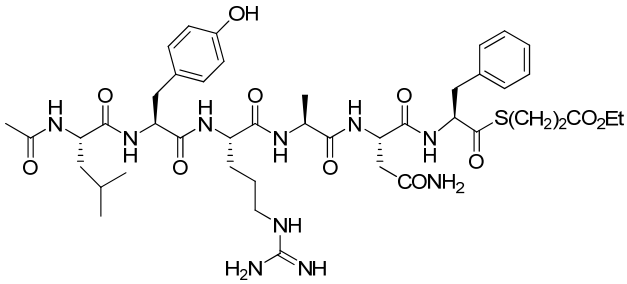
Ac-LYRANM-S(CH$_2$)$_2$CO$_2$Et (7)



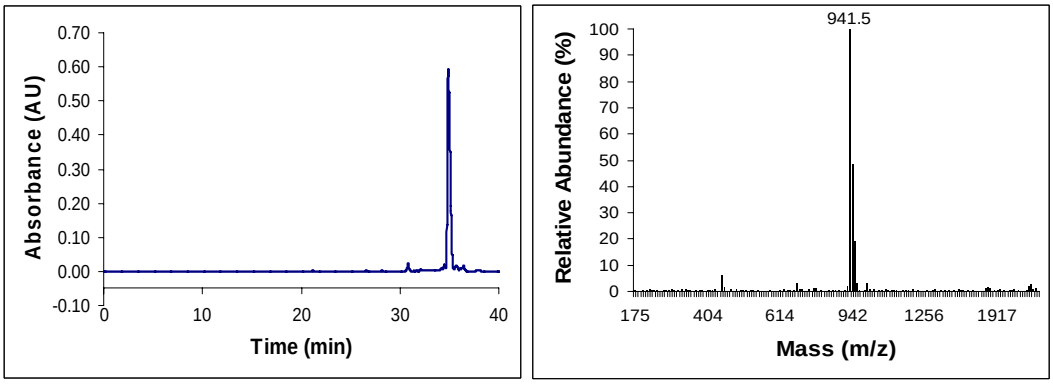
25 µmol of fully assembled Rink amide resin was treated with H-Met-S(CH$_2$)$_2$CO$_2$Et according to the general procedure described above. Yield: 20.6 mg, 90%; Calculated Mass $[M+H]^+$: 925.4, Mass Found 925.4; HPLC: t_R: 32.6 min (Gradient 0 to 50% B over 40 min.).



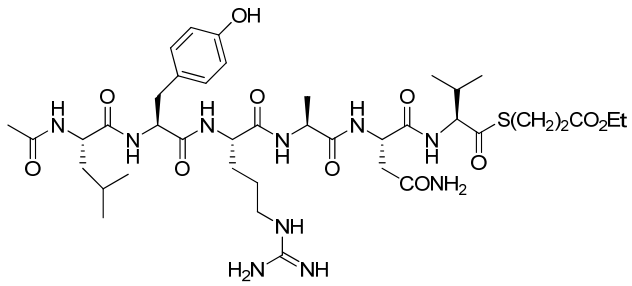
Ac-LYRANF-S(CH$_2$)$_2$CO$_2$Et (8)



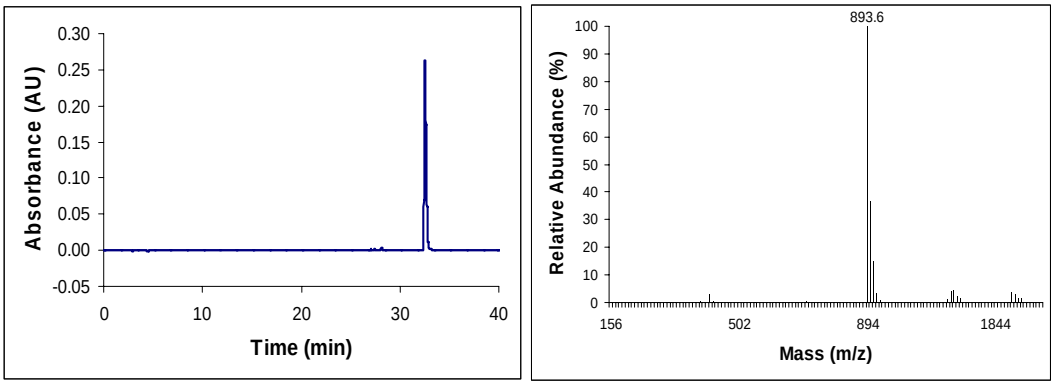
25 µmol of fully assembled Rink amide resin was treated with H-Phe-S(CH$_2$)$_2$CO$_2$Et according to the general procedure described above. Yield: 22.6 mg, 96%; Calculated Mass $[M+H]^+$: 941.5, Mass Found 941.5; HPLC: t_R: 34.9 min (Gradient 0 to 50% B over 40 min.).



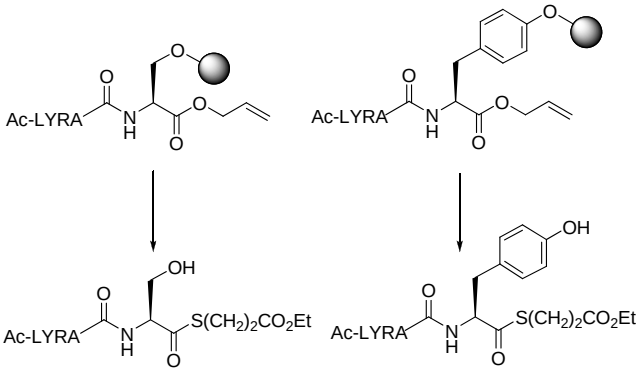
Ac-LYRANV-S(CH$_2$)$_2$CO$_2$Et (11)



25 µmol of fully assembled Rink amide resin was treated with H-Val-S(CH$_2$)$_2$CO$_2$Et according to the general procedure described above. Yield: 18.5 mg, 83%; Calculated Mass $[M+H]^+$: 893.5, Mass Found 893.6; HPLC: t_R: 32.5 min (Gradient 0 to 50% B over 40 min.).



General procedure for the direct thioesterification on solid support:

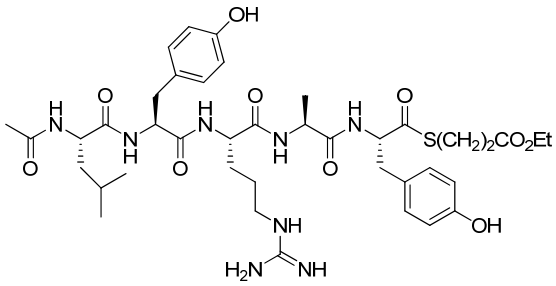


Bromo-(4-methoxyphenyl)methylpolystyrene resin (25 µmol) fully assembled with the desired peptide sequence was swollen in dry DCM for 30 min, followed by the addition of a solution of Pd(PPh$_3$)$_4$ (25 mg, 22 µmol) and PhSiH$_3$ (123 µl, 108 mg, 1 mmol) in dry DCM (2 mL). The resin was shaken for 1 h and the procedure was

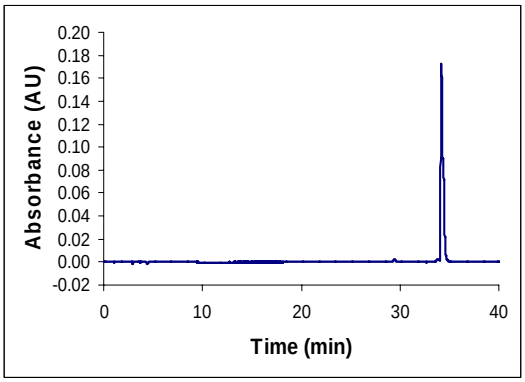
repeated once. Afterwards, the resin was washed DCM (x 10), DMF (x 5), DCM (x 5) and a solution of ethyl 3-mercaptopropionate (77 µl, 600 µmol, 24 equiv.), anhydrous HOBt (101 mg, 750 µmol, 30 eq.), DIEA (161 µl, 121 mg, 938 µmol, 37.5 eq.) and DIC (116 µl, 750 µmol, 30 eq.) in DCM/DMF (4:1 v/v, 1.5 ml) was added and the resin shaken for 1 h. This thioesterification step was repeated once before washing the resin with DCM (x 5), DMF (x 5) and DCM (x 10). *Cleavage*: A mixture of TFA, thioanisole, triisopropylsilane (TIS) and water (17:1:1:1 v/v/v/v) was added to the resin. After 2 h, the resin was washed with TFA (4x 4 mL) *Work-up*: The combined solutions were concentrated *in vacuo*. The residue was dissolved in water containing 0.1% TFA and purified by preparative HPLC (0 to 50% B over 40 min.) to afford the desired peptide thioesters as white fluffy solids which were analysed by HPLC (0 to 50% B over 40 min.) and ESI mass spectrometry.

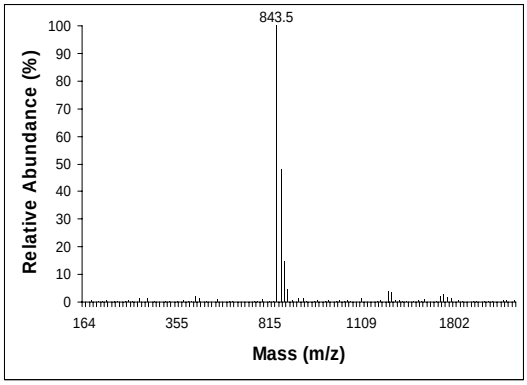
Analytical Data for peptide thioesters 9 and 10:

Ac-LYRAY-S(CH$_2$)$_2$CO$_2$Et (9)

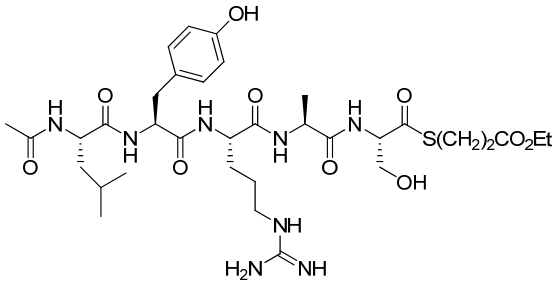


25 µmol of fully assembled Rink amide resin was treated with ethyl 3-mercaptopropionate according to the general procedure described above. Yield: 15.6 mg, 74%; Calculated Mass $[M+H]^+$: 843.4, Mass Found 843.5; HPLC: t_R: 34.2 min (Gradient 0 to 50% B over 40 min.).

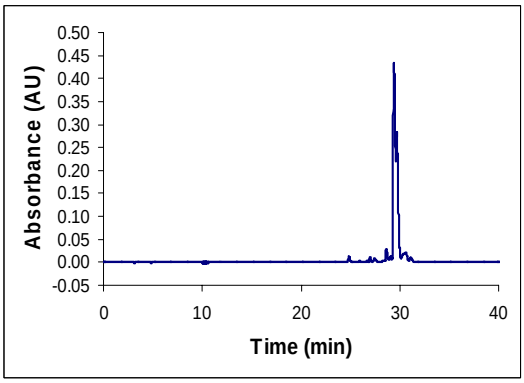


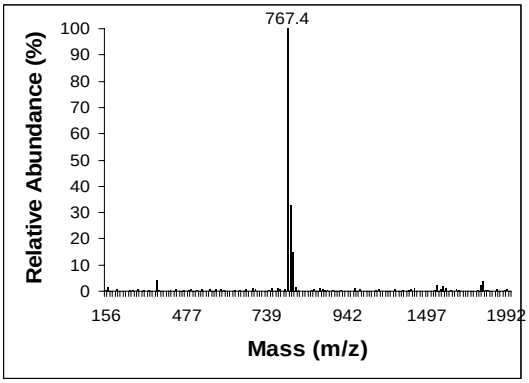


Ac-LYRAS-S(CH$_2$)$_2$CO$_2$Et (10)



25 µmol of fully assembled Rink amide resin was treated with ethyl 3-mercaptopropionate according to the general procedure described above. Yield: 13.3 mg, 70%; Calculated Mass $[M+H]^+$: 767.4, Mass Found 767.4; HPLC: t_R: 29.4 min (Gradient 0 to 50% B over 40 min.).





General procedure for the phosphate-assisted ligation

Phosphopeptides **1** and **2** (1.5 mg, approx. 2.18-2.22 µmol) were dissolved in 150µL of ligation buffer [4:1 v/v *N*-methyl-2-pyrrolidinone (NMP): 1.25 M guanidine hydrochloride, 0.2 M HEPES, pH = 8.5].[a] This solution was transferred to an

eppendorf tube containing the peptide thioester (**5** – **11**) (1.2 eq., 2.62-2.66 μmol) giving a final pH of 7.5. Thiophenol (2% by volume, 3 μL) was added and the reaction mixed gently. The ligation mixture was incubated at 37 °C with gentle mixing every 12 h until the reaction was confirmed to be complete by LC-MS. The ligation reactions were quenched by the addition of 0.1% TFA in water (0.6 mL). The products were purified by semi-preparative HPLC (0 to 50% B over 40 min.).

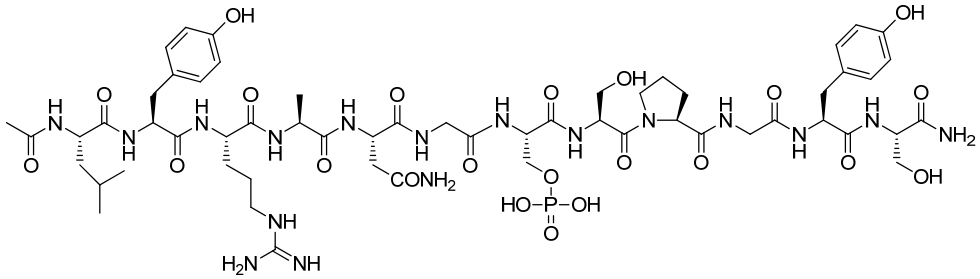
[a] An aqueous buffer containing 6 M Gn.HCl and 1 M HEPES was prepared and adjusted to pH 8.5 using 25% aqueous sodium hydroxide solution. The resulting solution (1 mL) was diluted with NMP (4 mL) to produce the final buffer for use in the phosphate assisted ligation reactions. Final concentrations are 1.25 M Gn. HCl and 0.2 M HEPES.

Kinetics of the phosphate-assisted ligations

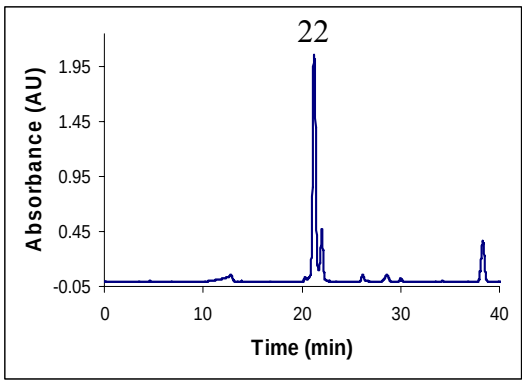
Phosphopeptides **1** and **2** and peptides **3** and **4** were reacted with peptide thioester **5** (Ac-LYRANG-S$(CH_2)_2CO_2NH_2$) under the ligation conditions described above. Aliquots of 10 μL were removed from the reaction mixture and quenched with water containing 0.1% TFA (90 μL). These aliquots were taken every 2 h for 12 h then every 12 h until peptide thioester **2** had been completely consumed (by peptide bond formation or hydrolysis). These samples were analyzed by analytical HPLC (0 to 50% B over 40 min.) and the percentage yield calculated by integrating the peaks (at λ = 280 nm) corresponding to starting peptides **1**-**4** and the ligated peptide/phosphopeptide product. NB: Since the resulting peptide/phosphopeptide products contain two tyrosine residues the integration was halved in order to calculate the ligation yield.

Analytical data for phosphate-assisted ligations

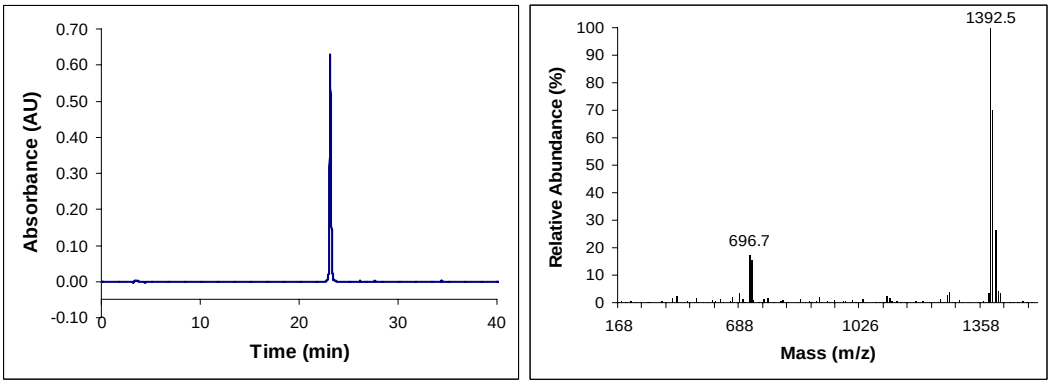
22 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **5**)



Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).

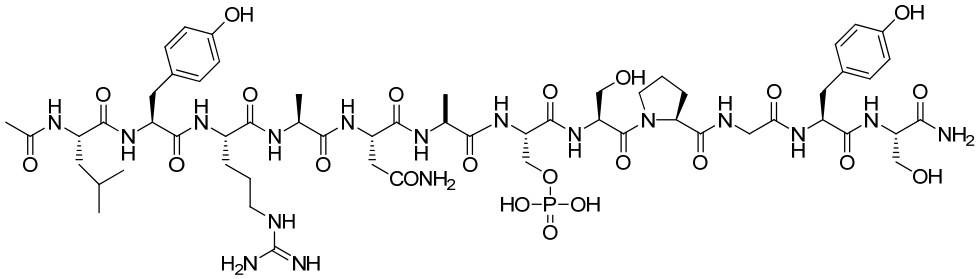


Analytical HPLC trace and ESI mass spectrometry data for **22**.

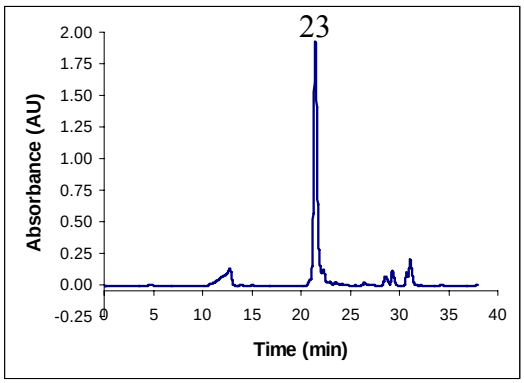


Yield: 2.8 mg, 91%; ESI (m/z): Calculated Mass $[M+H]^+$: 1392.4, Mass Found 1392.5; HPLC: t_R: 23.3 min (Gradient 0 to 50% B over 40 min.).

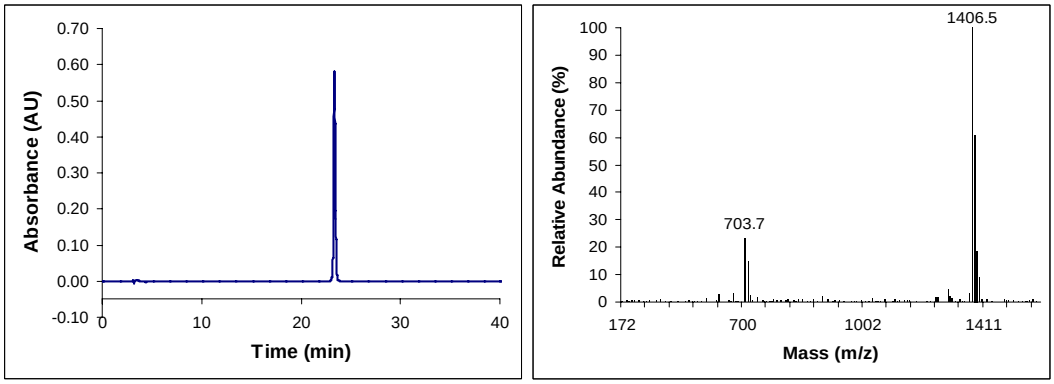
23 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **6**)



Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).

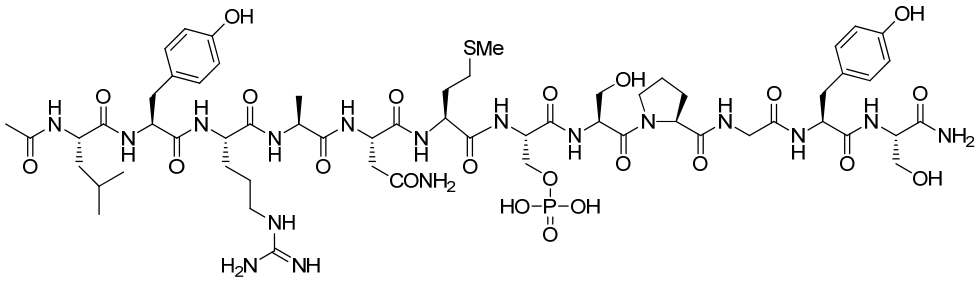


Analytical HPLC trace and ESI mass spectrometry data for **23**.

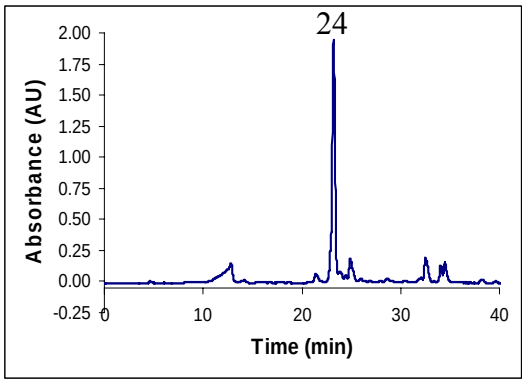


Yield: 2.8 mg, 90%; ESI (m/z): Calculated Mass $[M+H]^+$: 1406.4, Mass Found 1406.5; HPLC: t_R: 23.5 min (Gradient 0 to 50% B over 40 min.).

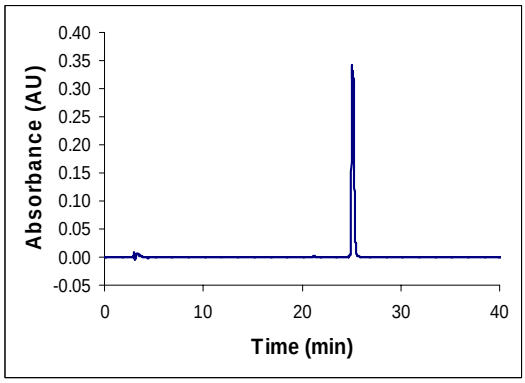
24 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **7**)

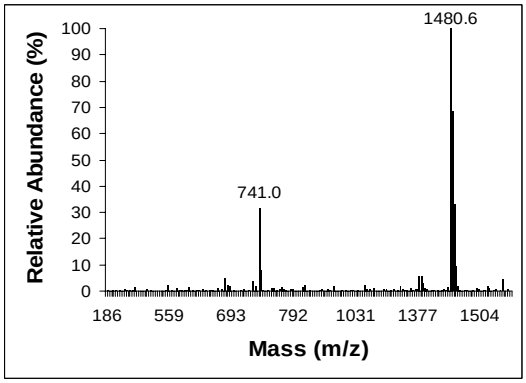


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



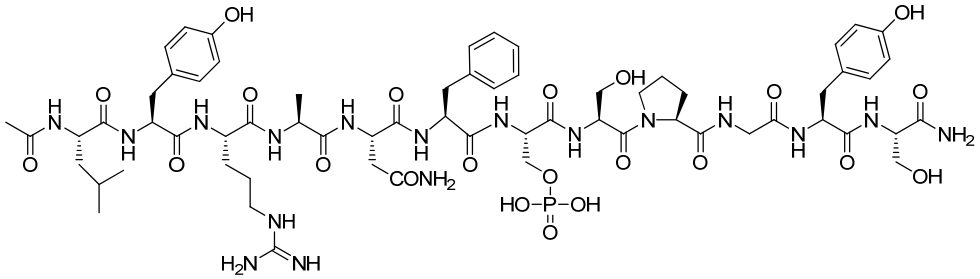
Analytical HPLC trace and ESI mass spectrometry data for **24**.



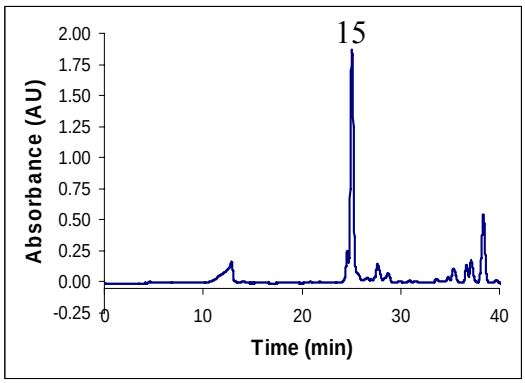


Yield: 2.3 mg, 71%; ESI (m/z): Calculated Mass $[M+H]^+$: 1466.5, Mass Found 1466.5; HPLC: t_R: 25.1 min (Gradient 0 to 50% B over 40 min.).

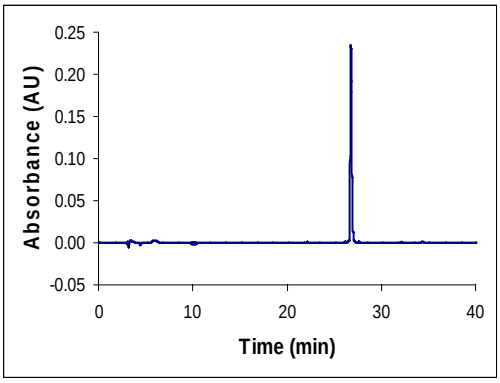
15 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **8**)

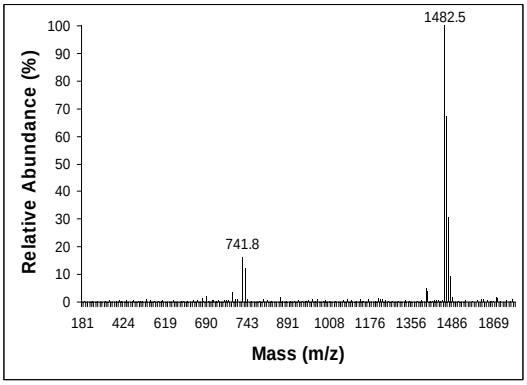


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



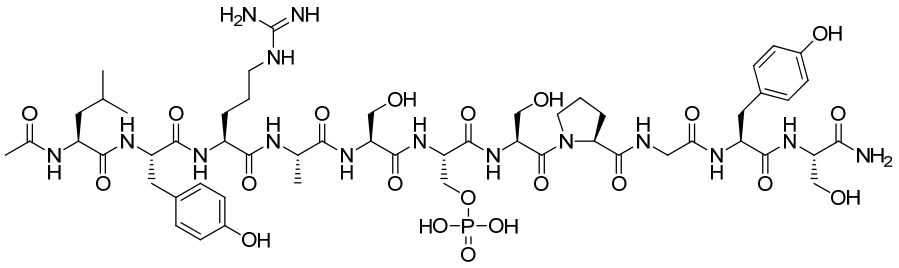
Analytical HPLC trace and ESI mass spectrometry data for **15**.



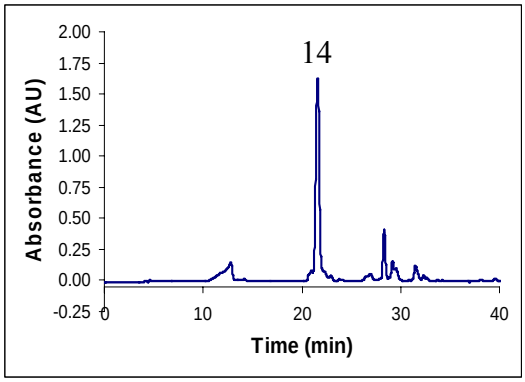


Yield: 2.72 mg, 82%; ESI (m/z): Calculated Mass $[M+H]^+$: 1482.5, Mass Found 1482.5; HPLC: t_R: 26.7 min (Gradient 0 to 50% B over 40 min.).

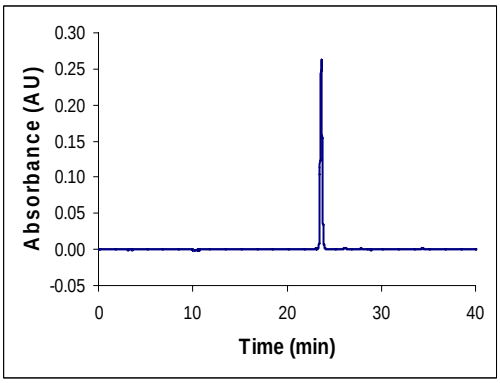
14 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **10**)

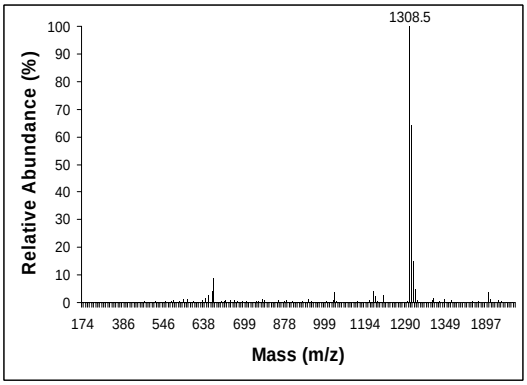


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



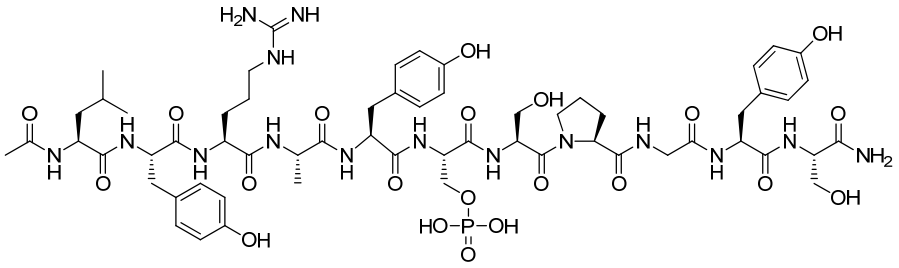
Analytical HPLC trace and ESI mass spectrometry data for **14**.



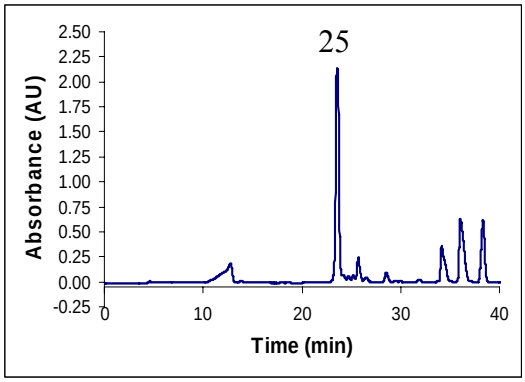


Yield: 1.8 mg, 62%; ESI (m/z): Calculated Mass $[M+H]^+$: 1308.3, Mass Found 1308.5; HPLC: t_R: 23.6 min (Gradient 0 to 50% B over 40 min.).

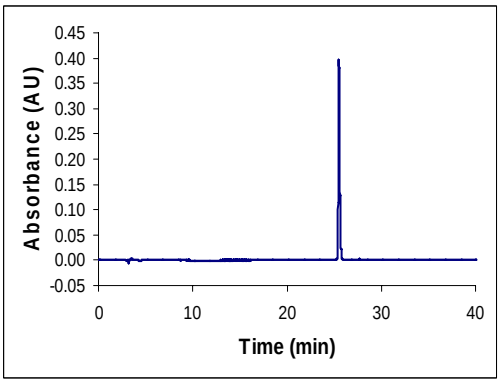
25 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **9**)

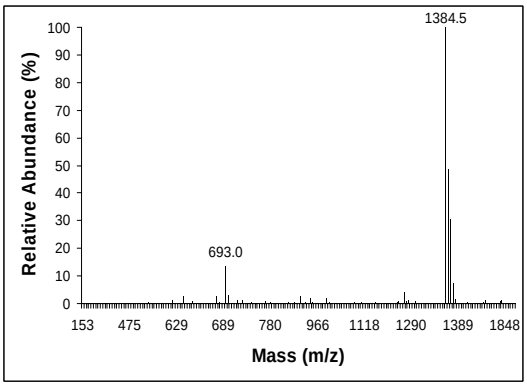


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



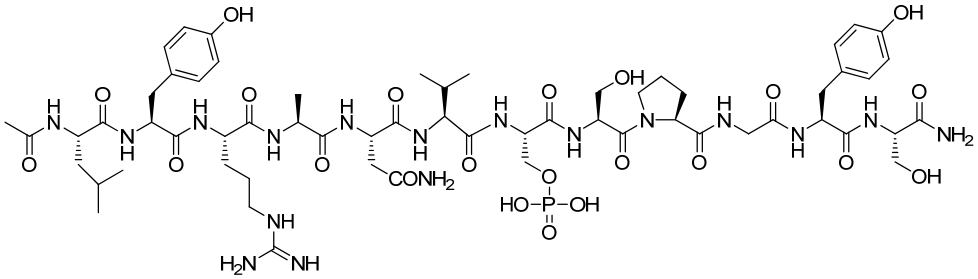
Analytical HPLC trace and ESI mass spectrometry data for **25**.



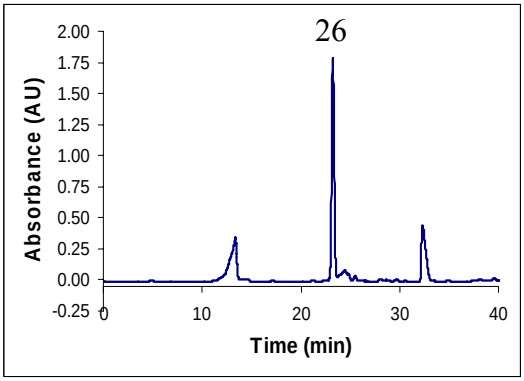


Yield: 2.7 mg, 82%; ESI (m/z): Calculated Mass $[M+H]^+$: 1384.4, Mass Found 1384.5; HPLC: t_R: 25.5 min (Gradient 0 to 50% B over 40 min.).

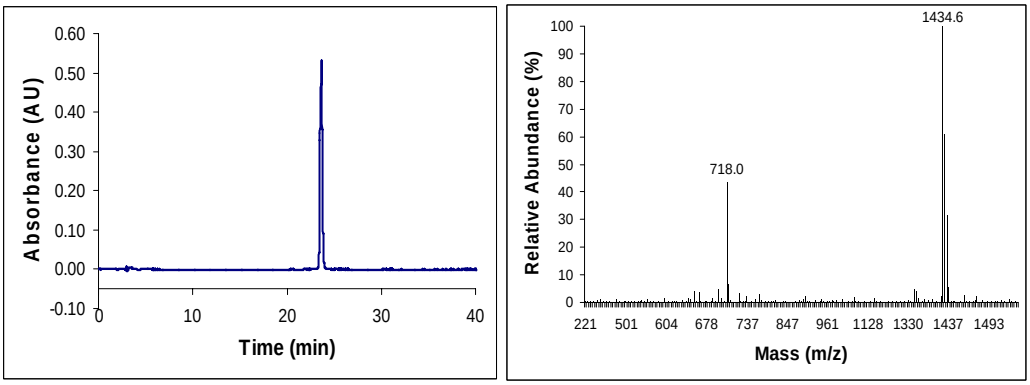
26 (Product of the phosphate-assisted ligation between phosphopeptide **1** and peptide thioester **11**)



Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).

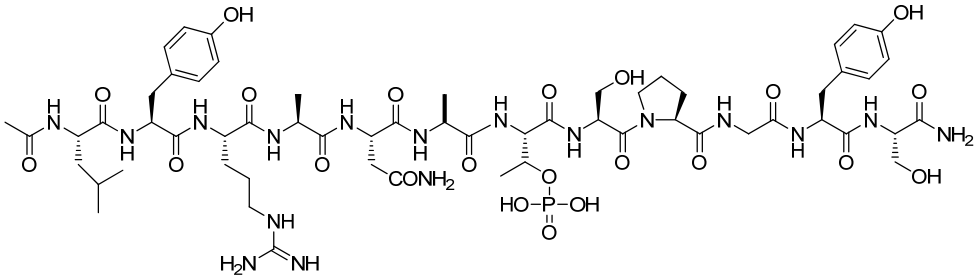


Analytical HPLC trace and ESI mass spectrometry data for **26**.

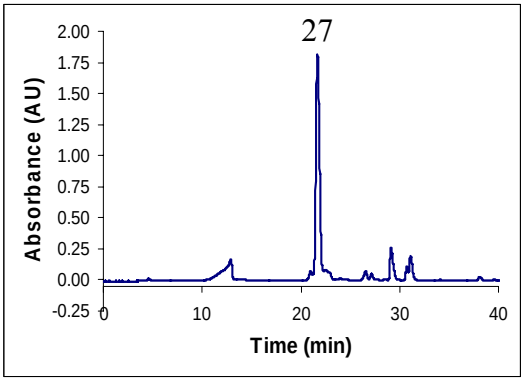


Yield: 1.5 mg, 47%; ESI (m/z): Calculated Mass $[M+H]^+$: 1434.5, Mass Found 1434.6; HPLC: t_R: 23.6 min (Gradient 0 to 50% B over 40 min.).

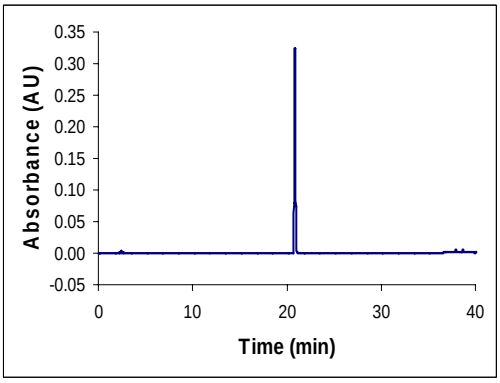
27 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **6**)

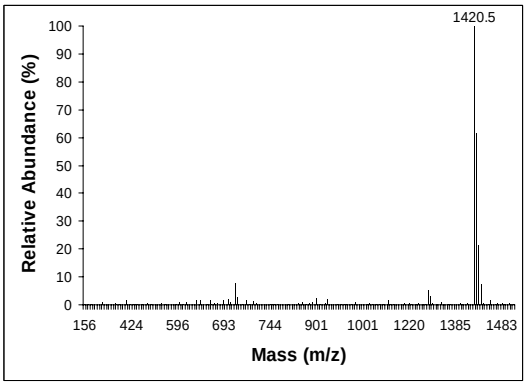


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



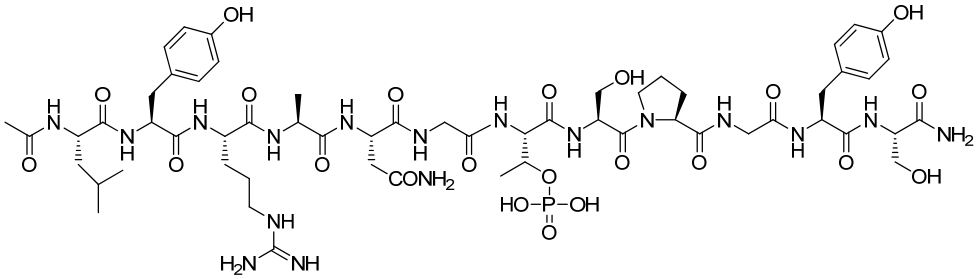
Analytical HPLC trace and ESI mass spectrometry data for **27**.



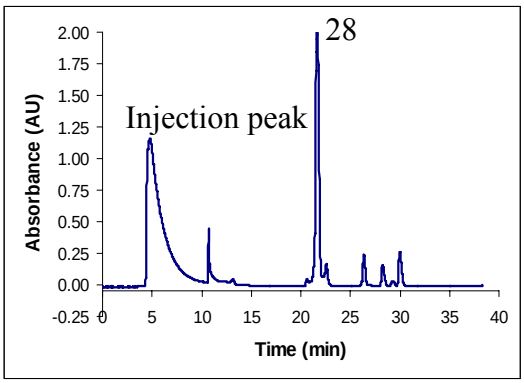


Yield: 1.9 mg, 61%; ESI (m/z): Calculated Mass $[M+H]^+$: 1420.4, Mass Found 1420.5; HPLC: t_R: 20.8 min (Gradient 0 to 50% B over 40 min.).

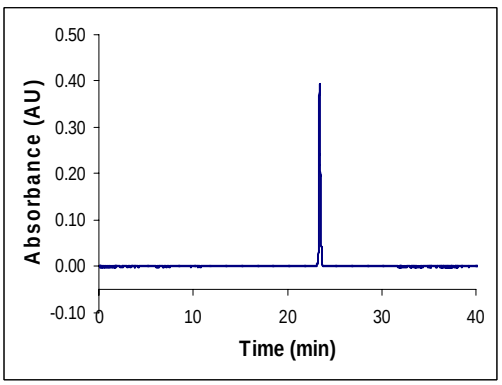
28 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **5**)

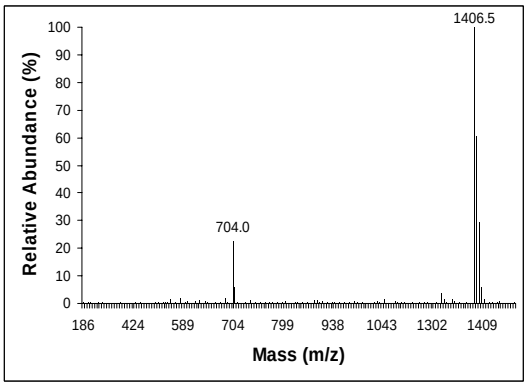


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



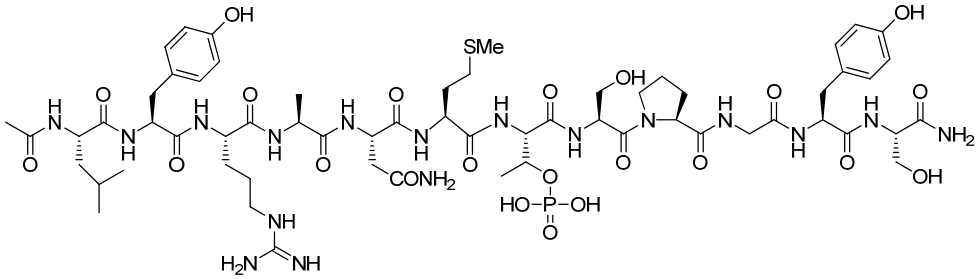
Analytical HPLC trace and ESI mass spectrometry data for **28**.



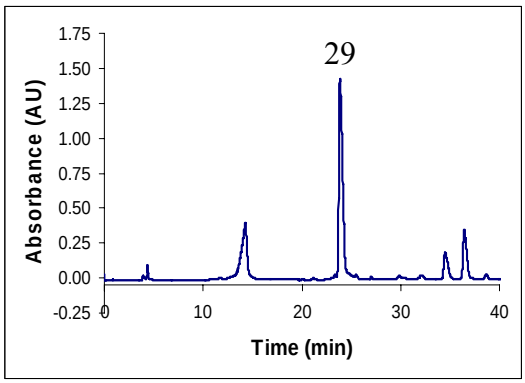


Yield: 2.9 mg, 95%; ESI (m/z): Calculated Mass $[M+H]^+$: 1406.4, Mass Found 1406.5; HPLC: t_R: 23.4 min (Gradient 0 to 50% B over 40 min.).

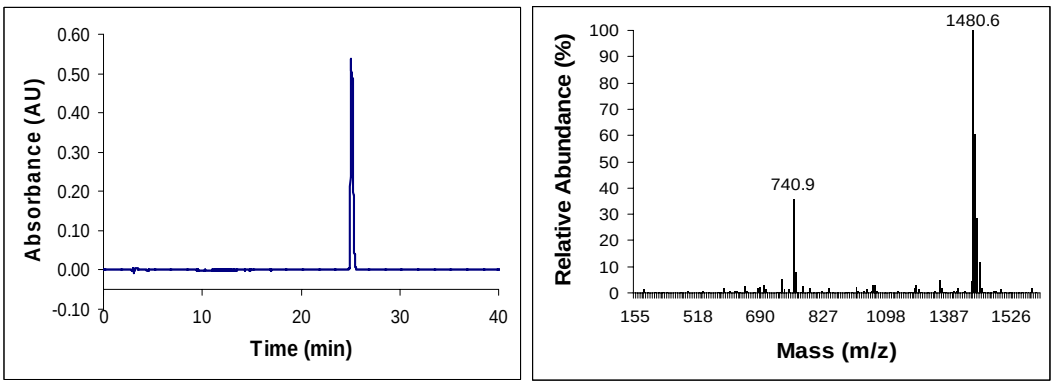
29 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **7**)



Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).

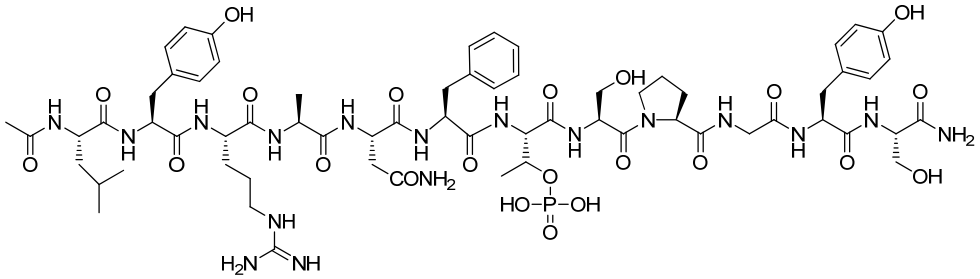


Analytical HPLC trace and ESI mass spectrometry data for **29**.

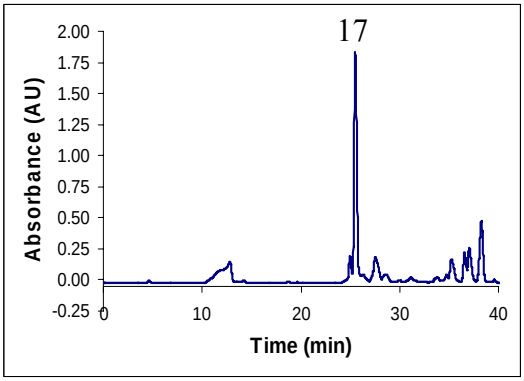


Yield: 1.6 mg, 50%; ESI (m/z): Calculated Mass $[M+H]^+$: 1480.5, Mass Found 1480.6; HPLC: t_R: 25.1 min (Gradient 0 to 50% B over 40 min.).

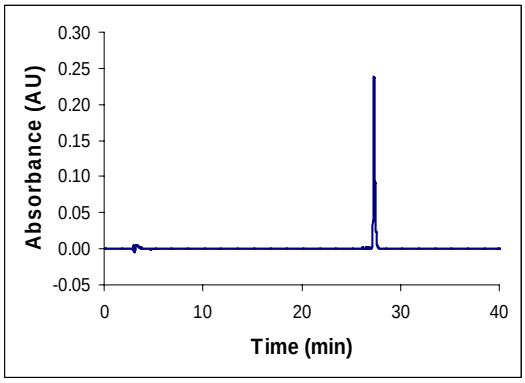
17 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **9**)

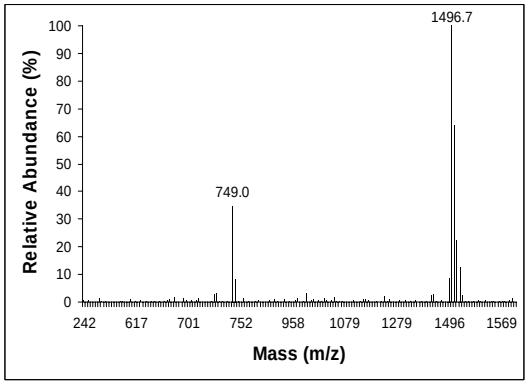


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



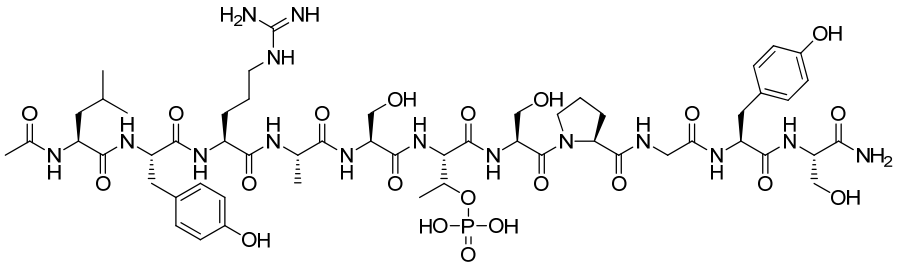
Analytical HPLC trace and ESI mass spectrometry data for **17**.



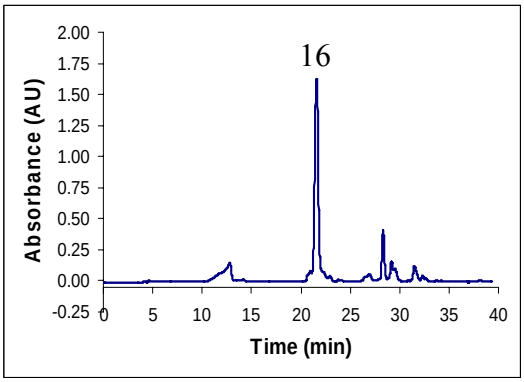


Yield: 1.7 mg, 52%; ESI (m/z): Calculated Mass $[M+H]^+$: 1496.5, Mass Found 1496.7; HPLC: t_R: 27.3 min (Gradient 0 to 50% B over 40 min.).

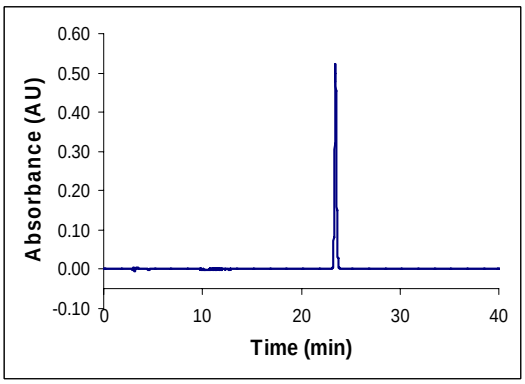
16 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **10**)

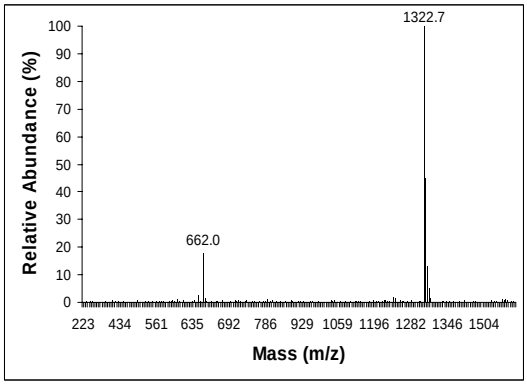


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



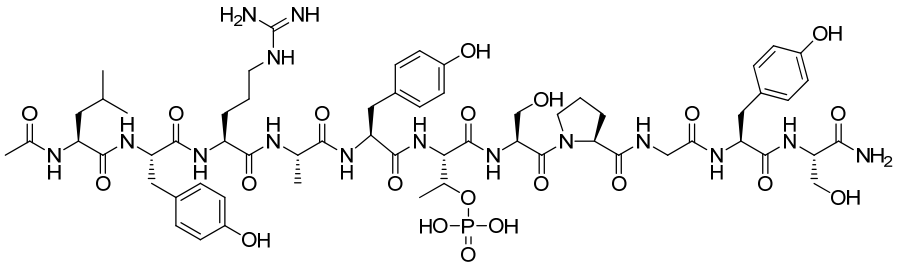
Analytical HPLC trace and ESI mass spectrometry data for **16**.



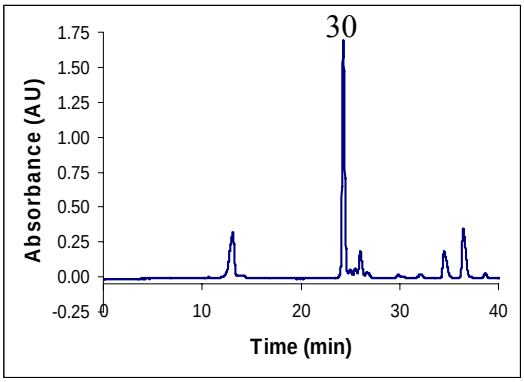


Yield: 1.8 mg, 62%; ESI (m/z): Calculated Mass $[M+H]^+$: 1322.3, Mass Found 1322.7; HPLC: t_R: 23.4 min (Gradient 0 to 50% B over 40 min.).

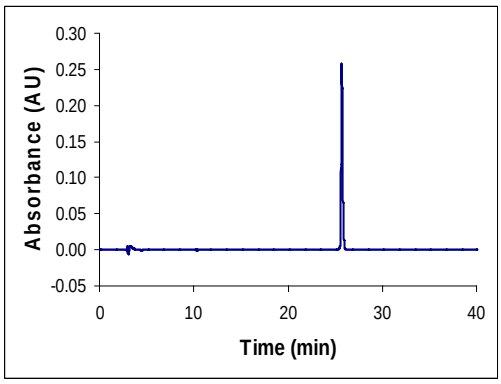
30 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **9**)

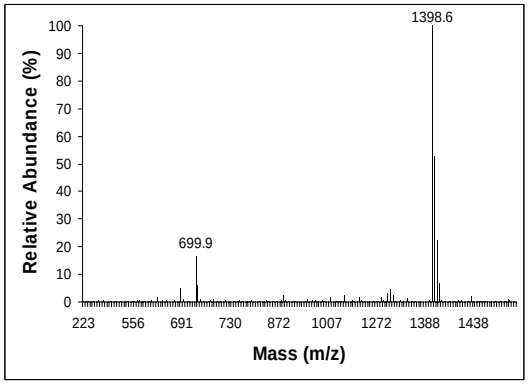


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



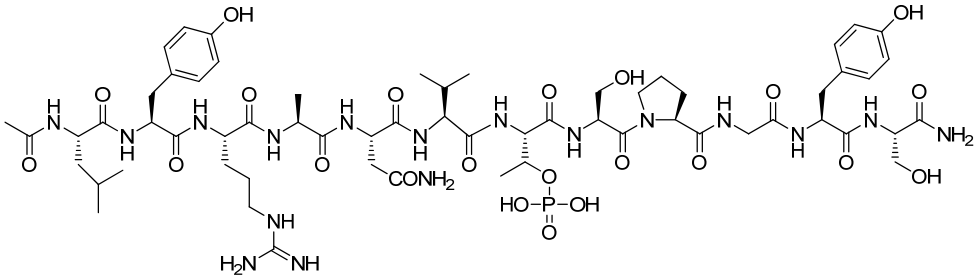
Analytical HPLC trace and ESI mass spectrometry data for **30**.



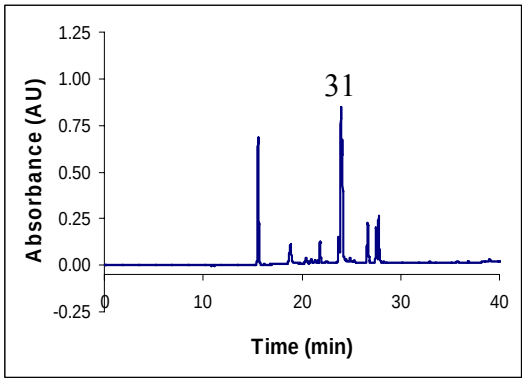


Yield: 2.1 mg, 90%; ESI (m/z): Calculated Mass $[M+H]^+$: 1398.4, Mass Found 1398.6; HPLC: t_R: 25.7 min (Gradient 0 to 50% B over 40 min.).

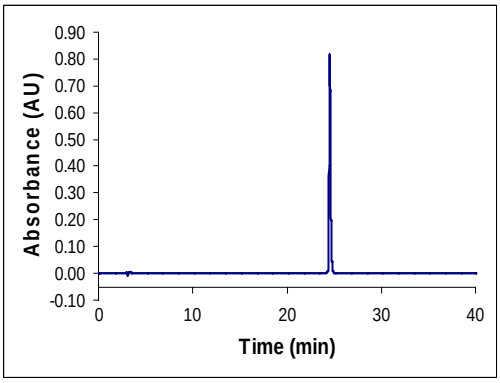
31 (Product of the phosphate-assisted ligation between phosphopeptide **2** and peptide thioester **11**)

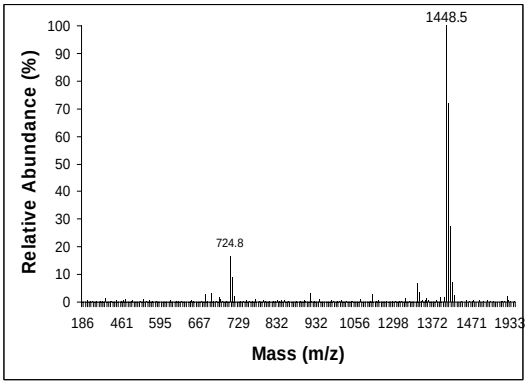


Crude semi-preparative HPLC trace of ligation reaction (λ = 280 nm).



Analytical HPLC trace and ESI mass spectrometry data for **31**.



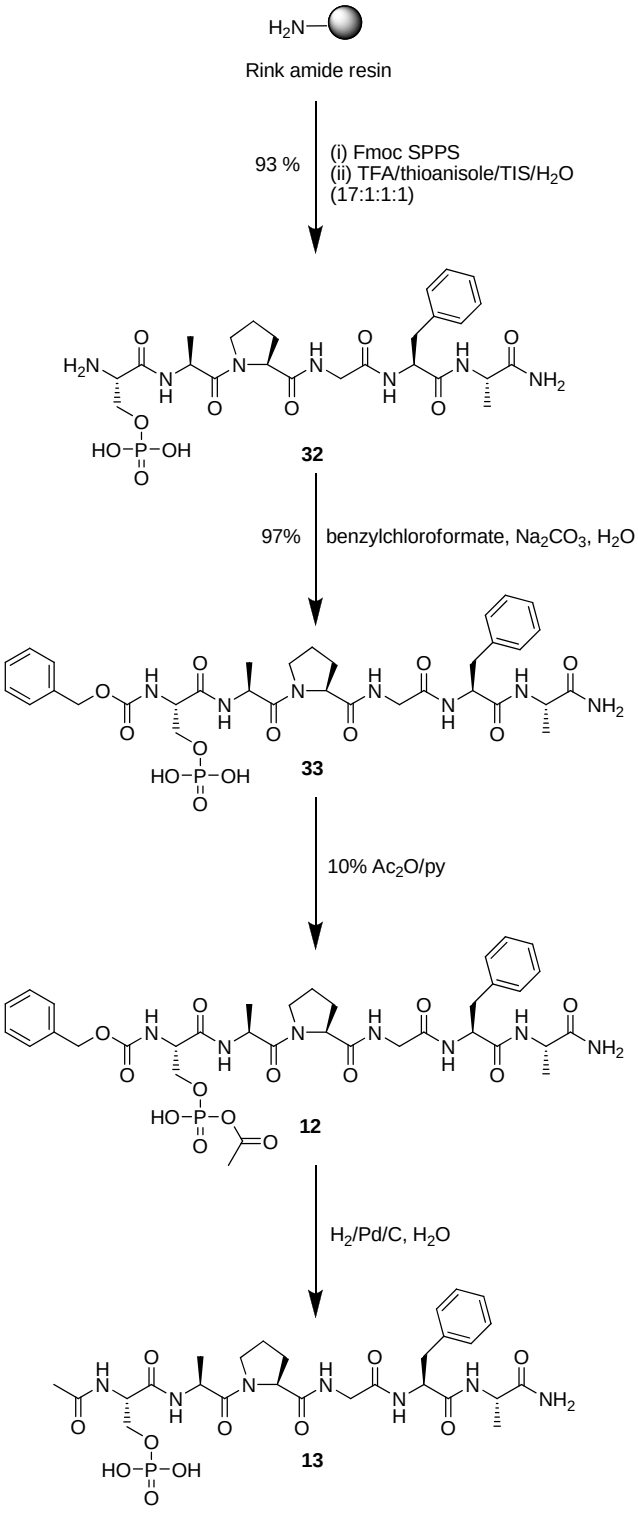


Yield: 1.5 mg, 47%; ESI (m/z): Calculated Mass $[M+H]^+$: 1448.5, Mass Found 1448.5; HPLC: t_R: 24.5 min (Gradient 0 to 50% B over 40 min.).

Hydrazine incubation of ligation products

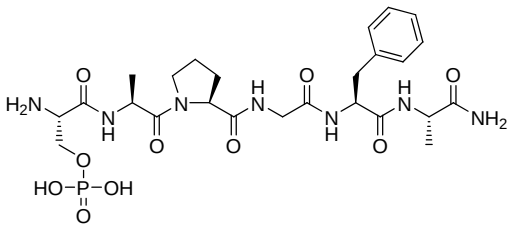
Phosphopeptides **14-17** and **22-31** were incubated with 10% hydrazine solution for 1 hour at room temperature. After this time analytical HPLC traces were obtained which showed identical retention times and mass spectra to those obtained for the product prior to treatment. This confirmed that an amide bond rather than the acyl phosphate had been generated in all ligation reactions.

Synthesis of acyl phosphate intermediate.

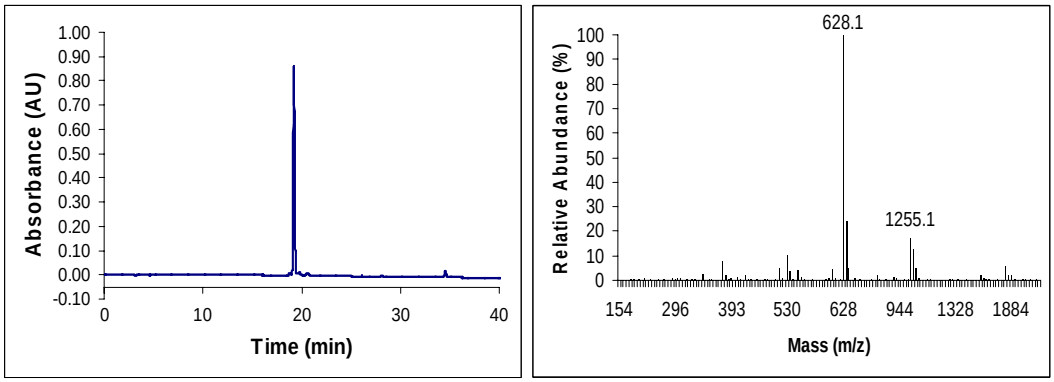


Analytical data for acyl phosphate 12 and N-acetylated product 13.

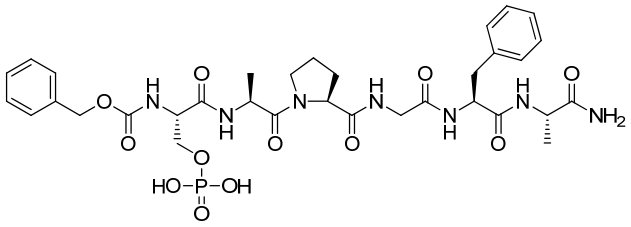
32



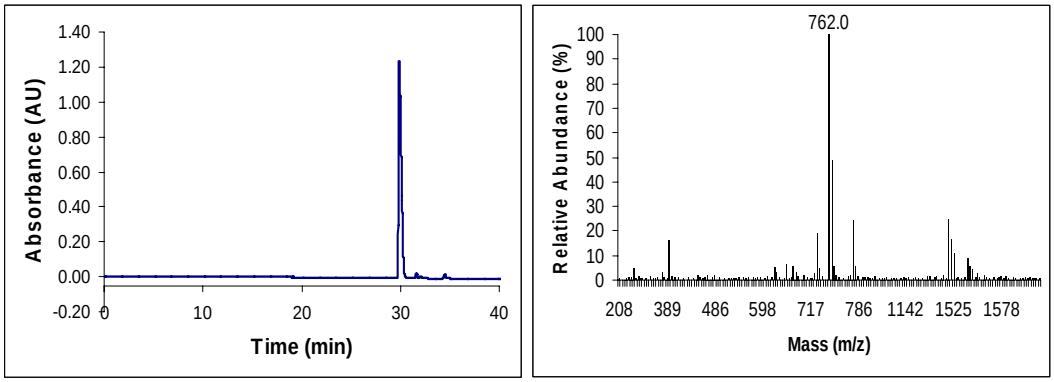
Phosphopeptide **32** was synthesised by Fmoc-strategy SPPS as described previously. 100 μM scale, Yield = 58 mg, 93%; ESI (m/z): Calculated Mass $[M+H]^+$: 628.2, Mass Found 628.1; HPLC: t_R: 19.20 min (Gradient 0 to 50% B over 40 min.).



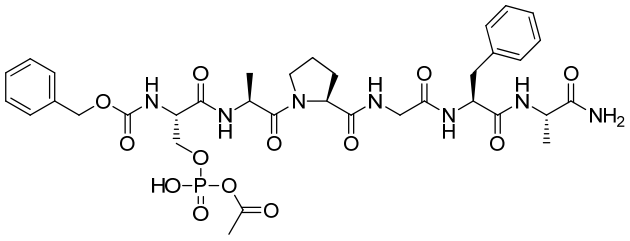
33



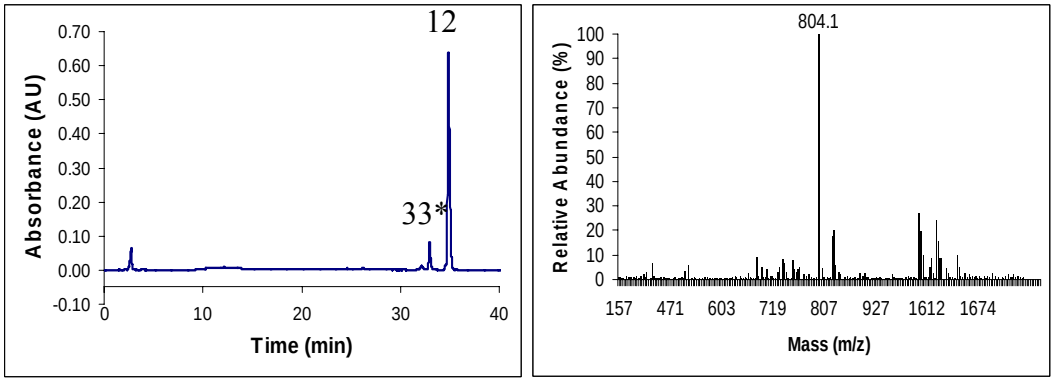
To a solution of **32** (20 mg, 27 μmol) in water (100 μL) was added benzylchloroformate (0.01 ml, 54 μmol) and sodium carbonate (4 mg, 10.4 μmol). The reaction was shaken for 12 hours and purified by HPLC to yield the Cbz-protected phosphorylated peptide. Yield = 20 mg, 97 %; ESI (m/z): Calculated Mass $[M+H]^+$: 762.3, Mass Found 762.0; HPLC: t_R: 29.88 min (Gradient 0 to 50% B over 40 min.).



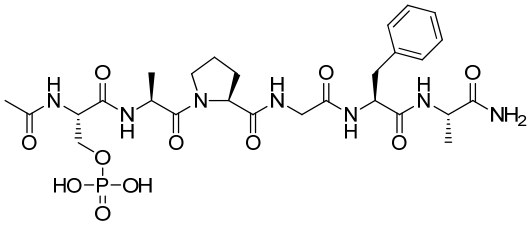
12



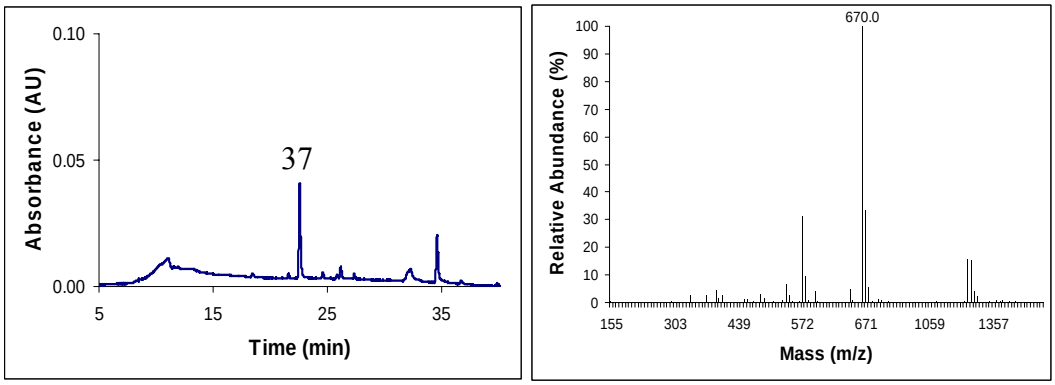
A solution of **33** (2 mg, 2.7 μmol) in 10% acetic anhydride in pyridine (500 μL) was shaken for 1 hour, the pyridine was removed under a steady stream of nitrogen and the crude product purified by HPLC. ESI (m/z): Calculated Mass $[M+H]^+$: 804.3, Mass Found 804.1; HPLC: t_R: 34.9 min (Gradient 0 to 50% B over 40 min.). * NB: acyl phosphate **12** was very unstable and rapidly hydrolysed back to **33** at pH 7 it was used as a solution in the next step without lyophilisation.



13



To a solution of **12** in water was added palladium on carbon (~10 mg) and the reaction placed under an atmosphere of hydrogen. The reaction was stirred for 20 minutes at which point the product was purified via analytical HPLC. The N-acetylated product was verified by incubation with 5% hydrazine for 2 h. The incubated product was re-analysed by HPLC and LC-MS which confirmed the formation of **13** (0.75 mg, ~40%). ESI (m/z): Calculated Mass $[M+H]^+$: 670.3, Mass Found 670.0; HPLC: t_R: 22.6 min (Gradient 0 to 50% B over 40 min.).



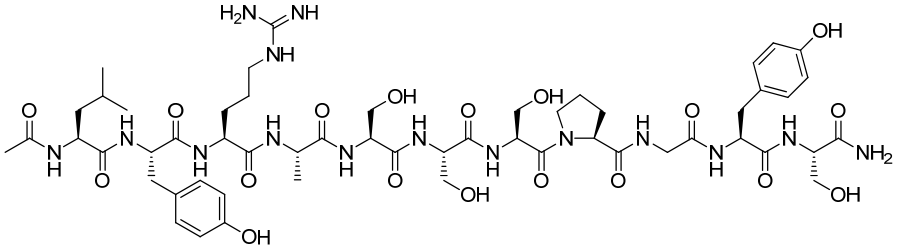
General procedure for dephosphorylation of phosphopeptide ligation products

Phosphopeptide ligation products (1.0 mg) were dissolved 50 mM Tris buffer at pH 8.6 (50 μL). Alkaline phosphatase (Sigma: Alkaline phosphatase from bovine intestinal mucosa, P5521) (7.5 units) was added and the reactions incubated at 37 ^oC. The dephosphorylation reactions were monitored by LC-MS and took between 2 and 24 h to reach completion.*

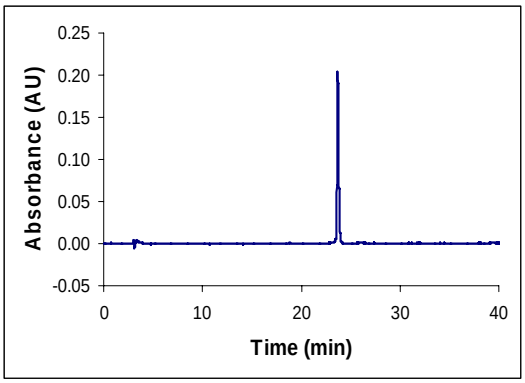
*NB: peptides containing phosphorylated threonine residues were slower to dephosphorylate with alkaline phosphatase

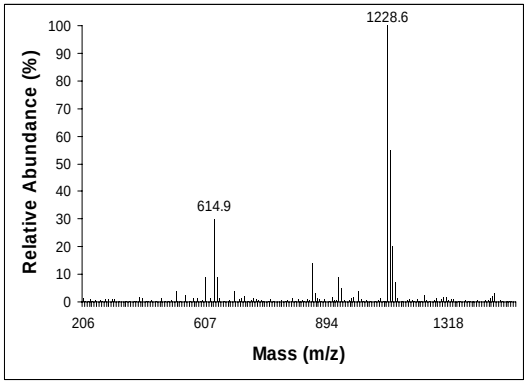
Analytical data for dephosphorylation reactions

18



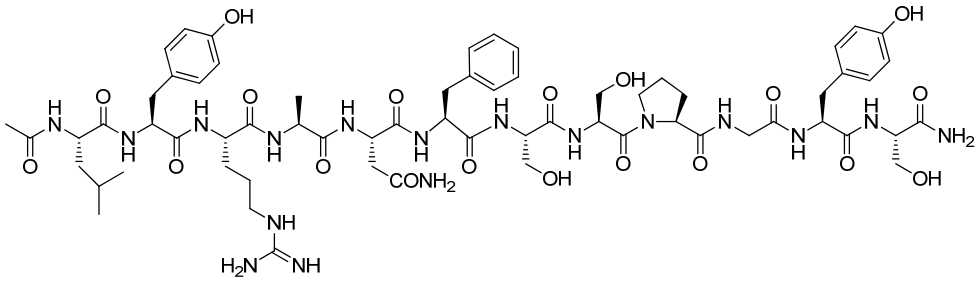
Analytical HPLC trace and ESI mass spectrometry data for **18**.



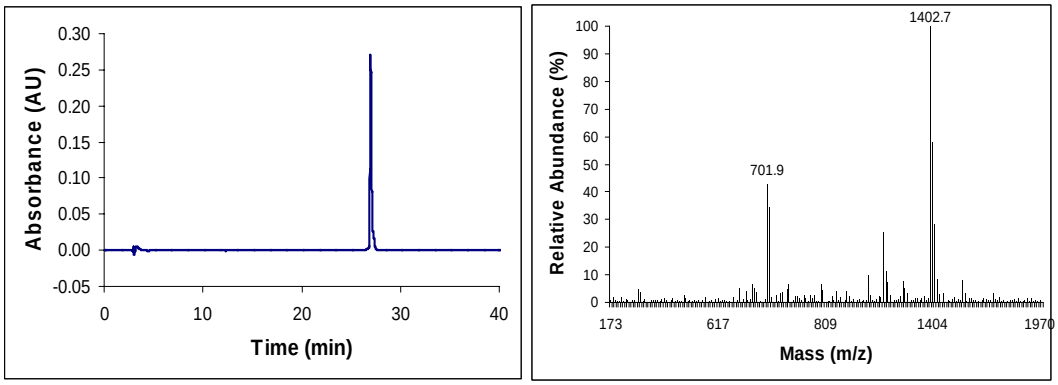


Phosphopeptide **14** was dephosphorylated using the conditions described above to afford **18** as a white fluffy solid. Yield: 98%; ESI (m/z): Calculated Mass $[M+H]^+$: 1228.3, Mass Found 1228.6; HPLC: t_R: 23.7 min (Gradient 0 to 50% B over 40 min.).

19

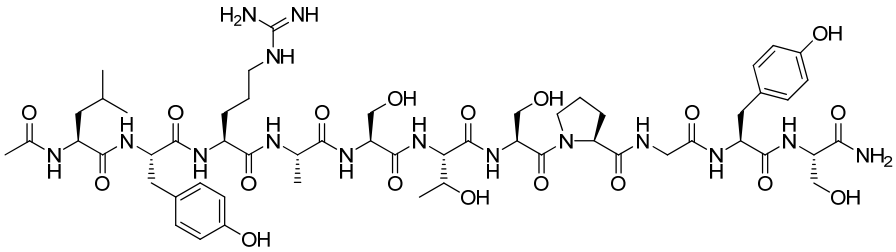


Analytical HPLC trace and ESI mass spectrometry data for **19**.

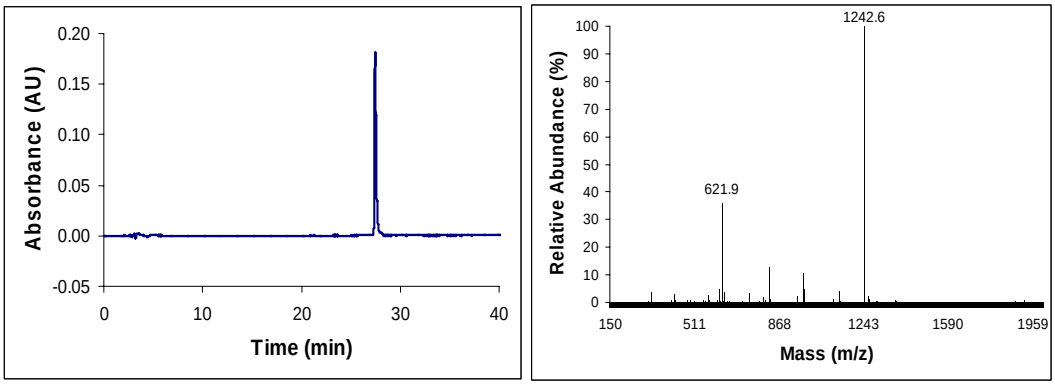


Phosphopeptide **15** was dephosphorylated using the conditions described above to afford **19** as a white fluffy solid. Yield: 74%; ESI (m/z): Calculated Mass $[M+H]^+$: 1402.5, Mass Found 1402.7; HPLC: t_R: 27.0 min (Gradient 0 to 50% B over 40 min.).

20

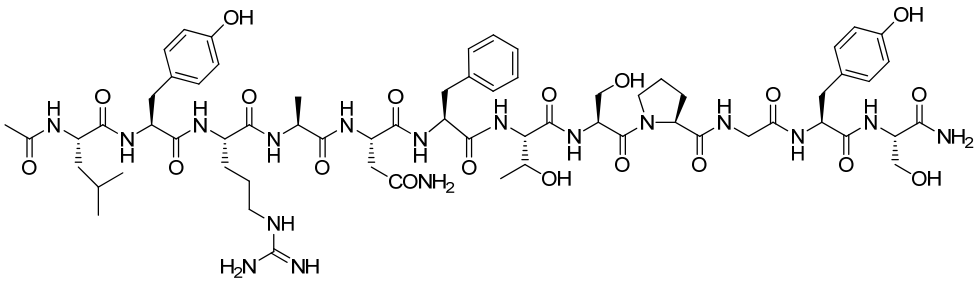


Analytical HPLC trace and ESI mass spectrometry data for **20**.

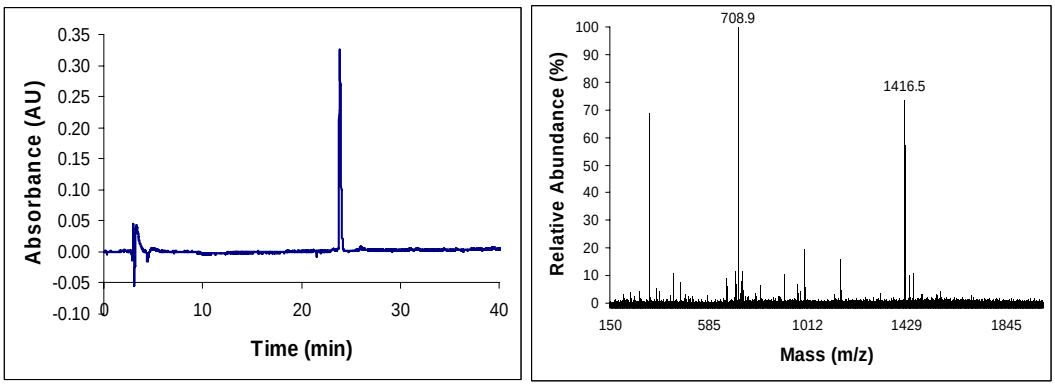


Phosphopeptide **16** was dephosphorylated using the conditions described above to afford **20** as a white fluffy solid. Yield: 85%; ESI (m/z): Calculated Mass $[M+H]^+$: 1242.3, Mass Found 1242.6; HPLC: t_R: 27.5 min (Gradient 0 to 50% B over 40 min.).

21



Analytical HPLC trace and ESI mass spectrometry data for **21**.



Phosphopeptide **17** was dephosphorylated using the conditions described above to afford **21** as a white fluffy solid. Yield: 89%; ESI (m/z): Calculated Mass $[M+H]^+$: 1416.5, Mass Found 1416.5; HPLC: t_R: 23.9 min (Gradient 0 to 50% B over 40 min.).

References

1) Ficht, S.; Payne R. J.; Guy R. T.; Wong C. H. *Chem. Eur. J.* **2008**, 14, 3620-3629.