Synthesis and Evaluation of Non-Hydrolyzable Phospho-Lysine Peptide Mimics

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1. Supplementary Figures

1.1 Figure S1

Side products occurred during SPPS using building block 3 and corresponding yields. While the desired peptide 12 was obtained, a considerable P–N bond hydrolysis was observed, as well as another side product, in which the detected mass would correspond to a Tyr addition and subsequent acetylation at the lysine side-chain.

\[
\begin{align*}
\text{SPPS using monomer 3} & \\
\xrightarrow{\text{TFA cleavage}} & \\
\text{12} & \quad \text{14\%} \\
+ & \\
\text{P–N hydrolysis} & \quad \text{11\%} \\
+ & \\
\text{Tyr addition} & \quad \text{7\%}
\end{align*}
\]

\[12 : \text{P–N hydrolysis} : \text{Tyr addition} = 1.3 : 1 : 0.5\]
1.2 Figure S2

Treatment of peptides 8a-c with alkaline phosphatase (ALP). Figure S2 shows the amount of enzymatically hydrolyzed substrate, calculated by subtraction of detected inorganic phosphate (Pᵢ) in blank reactions (without enzyme) from the reactions with enzyme added. Herein, we observed dephosphorylation for both peptides 8b and 8c, which indicated susceptibility of the phosphate analogue towards phosphatases. As expected, no Pᵢ was released from phosphonate 8a during incubation with ALP.
1.3 Figure S3

NMR titration of peptide 8b. Herein, the signals extracted from the $^1$H-$^{31}$P-HMBC experiments in distinct pH values are shown. Conditions: 1 mM peptide in 40 mM KCl in H2O + 10%D2O was measured at 278 K in a 600MHz ($^1$H frequency) spectrometer at pH values from 2 to 11.

A. Overlay of $^{31}$P NMR 1D spectra at different pH values.

B. Extracted correlation signals between $\varepsilon$-protons and phosphorus of 8b from $^1$H-$^{31}$P-HMBC NMR experiments.
1.4 Figure S4

Graphical visualizations of NMR measurements of $^1$H chemical shifts of phosphonate 8a (ζ-protons), phosphate 8b (ε-protons) and phosphoramidate 8c (ε-protons).
2. General Information

2.1 Reagents and Solvents

Reagents and solvents were, unless stated otherwise, commercially available as reagent grade and did not require further purification. Chemicals were purchased either from Sigma-Aldrich Chemie GmbH (Munich, Germany) or TCI (TCI Deutschland GmbH, Eschborn, Germany). Resins, coupling reagents and amino acids suitable for SPPS were purchased either from IRIS Biotech GmbH (Marktrewitz, Germany) or Novabiochem© (Merck KGaA, Darmstadt, Germany). Aliquots from bovine alkaline phosphatase (ALP, Sigma-Aldrich) were kindly provided by Prof. Fiedler. All water- and air-sensitive reactions were performed under Schlenk conditions.

2.2 Peptide Synthesis

Peptides were prepared using the Fmoc solid phase strategy by manual peptide synthesis in 8 mL Extract CleanTM reservoirs (Grace Davison Discovery Sciences, Deerfield, Illinois, USA) of 40 mL reactors (Activotec, Cambridge, UK) both equipped with fitting teflon frits.

Coupling of first amino acid on Rink amide resin

Swelling: 3.5 g Rink Amide resin (Novabiochem, 100-200 mesh, Fmoc on, average loading: 0.73 mmol x g⁻¹) were swollen in DMF for 45 min.

Fmoc deprotection: After 2x 10 min shaking, each with 10 mL of piperidine (Pip): DMF (1:4, v/v) the resin was washed with 5x DMF, 5x DCM, 5x DMF.

Coupling: A 0.1 M solution of 0.4 eq. (1 mmol) Fmoc-protected Glycine, 0.4 eq (1 mmol) PyBop and 0.8 eq. (2 mmol) DIPEA in DMF was added to the resin. After 2 h of shaking at r.t., the resin was washed (5x DMF, 5x DCM, 5x DMF).

Capping: A solution of Ac₂O:2,6-lutidine: DMF (5:6:89, v/v/v, 10 mL) was added and the mixture shaken for 10 min. The resin was washed (5x DMF, 10x DCM) and dried completely under reduced pressure. The loading was determined photometrically as described in the UV/Vis spectroscopy section. For every peptide synthesis described herein, an Fmoc-Gly pre-loaded resin 17 was prepared with a determined loading of 0.4385 µmol·mg⁻¹.
Manual peptide synthesis

Swelling: The corresponding amount of resin for a 100 μmol scale was swollen in DMF (2000 μL) for 30min.

Fmoc deprotection: Pip:DMF (1:4, v/v, 1000 μL) was added to the resin. After 5 min, the solution was discarded and another portion of Pip:DMF (1:4, v/v, 1000 μL) was added to the resin. After 5 min, the solution was discarded and the resin washed with DMF, DCM, DMF (3x 1000 μL each).

Coupling: Aas were dissolved together with HCTU (500 μmol, 5 eq.) and Oxyma (500 μmol, 5 eq.) to a 0.2M solution in DMF. Directly before adding the mixture to the resin, DIPEA (1000 μmol, 10 eq.) was added. The resulting reaction mixture was shaken for 45 min at r.t., the resin then filtered and washed with DMF, DCM, DMF (3x 1000 μL each). Fmoc-Lys(N3)-OH, Fmoc-hCys(PO(OBn)2)-OH 1, Fmoc-Nle(OPO(OH)(OBn))-OH 2, Fmoc-Lys(PO(OTc)2)-OH 3 were incorporated by using 2 eq. AA, 1.95 eq. HATU and 4 eq. DIPEA in DMF and reacting the mixture for 2 h at r.t.

Acetylation: N-terminal acetylation was performed by treating the resin with a mixture of Ac2O:2,6-lutidine: DMF (5:6:89, 1 mL) for 10 min at r.t., after which the resin was washed with DMF, DCM, DMF (3x 1000 μL each).

Final cleavage: The resin was either washed with DCM (10x 1000 μL) or used dry and treated with 4 mL of the cleavage cocktail (TFA: TIS: H2O – 95:2.5:2.5, v/v/v) for 2 h. Peptides containing the hCys-derivative 1 were treated for 105 min with the standard cleavage cocktail, before a solution of 60 μL EDT and 300 μL TMSBr was added and the mixture shaken for further 15 min. The resin was filtered off, the TFA filtrate collected in a 10-fold excess of deep-frozen Et2O and let sit for precipitation in the freezer. After at least 15 min, the mixture was centrifuged, the solution decanted, the precipitate dried under nitrogen and re-dissolved in ACN/H2O for UPLC analysis and preparative HPLC.

2.3 Analytical UPLC

UPLC®-UV traces for peptides and small molecules were obtained on an ACQUITY H-class instrument (Waters Corporation, Milford, Massachusetts, USA) equipped with an ACQUITY UPLC®-BEH C18 1.7 &m, 2.1x50 mm column (Waters Corporation), applying a flow rate of 0.6 ml x min⁻¹ and using eluents A (99.9% H2O, 0.1% TFA) and B (99.9% ACN, 0.1% TFA) in the corresponding linear gradient. UPLC-UV chromatograms were recorded at 220 nm.

Gradients: I 5% to 95% B in 13 min  
II 0.5% to 60% B in 13 min
High resolution masses were recorded on an ACQUITY H-class instrument (Waters Corporation) equipped with an ESI-MS Xevo® G2-XS QTof spectrometer (Waters Corporation).

2.4 TLC analysis

The thin layer chromatography (TLC) was performed on silica gel plates with fluorescence indicator F254 (Merck KGaA). Detection was performed at 254 or 366 nm. Compounds without any chromophore were stained with any of staining solutions such as potassium permanganate solution, ninhydrin or vanillin reagent.

2.5 Purification

Peptidic substrates were purified by preparative, semi-preparative or analytical HPLC, performed either on a Gilson PLC 2020 system (Gilson Inc., Middleton, Wisconsin, USA), a Shimadzu Prominance 20A system or a Shimadzu Prominance 8A system (both Shimadzu Corporation, Kyoto, Japan) equipped with columns as followed: preparative column – Nucleodur C18 HTec, 5 μm, 250x32 mm; semi-preparative column – Nucleodur C18 HTec, 5 μm, 250x21 mm; analytical column – Nucleodur C18 HTec, 5 μm, 250x10 mm (all columns purchased from Macherey-Nagel, GmbH & Co. KG, Düren, Germany). Eluents A (99.9% H₂O, 0.1% TFA) and B (B I: 99.9% ACN, 0.1% TFA or B II: 9.9% ACN, 20% H₂O, 0.1% TFA) were applied in the corresponding linear gradient. Peak detection was performed at 220 nm. Small molecules were purified by silica gel column chromatography (VWR Chemicals, Normasil 60 Å, 40–63 μm). The samples were applied pre-absorbed on silica gel or, if liquid, directly diluted with suitable solvents.

2.6 NMR

NMR spectra were recorded either with a Bruker AV III HD 300MHz spectrometer or a Bruker AV III600MHz spectrometer (both Bruker Corporation, Billerica, Massachusetts USA) at ambient temperature if not stated differently. The chemical shifts for proton signals (¹H) are reported in ppm relative to the shift of tetramethylsilane. The chemical shifts for phosphorous signals (³¹P) are reported relative to the signal of phosphoric acid (H₃PO₄) using indirect calibration.
**NMR titration experiments**

Typically, samples were dissolved in 40 mM KCl to a concentration of 1 mM. The solutions were immediately cooled in an ice-water bath. The pH meter was calibrated at 20 °C; however, all sample readings were performed in the ice-water bath. The pH of the samples was measured before and after the NMR experiment and agreed to within 0.01 pH unit. The pH values used for calculations are the average of the two readings and are not corrected for the temperature. Solutions were then transferred into a 5 mm sample tubes and sealed capillaries filled with D$_2$O were added. The samples were measured sequentially using an automatic sample changer and were thus kept a room temperature for most of the time, the temperature for the experiments was adjusted only after insertion into the magnet.

Experiments were performed at 278 K on a AV-III-600 NMR-spectrometer (600 MHz $^1$H frequency, Bruker Biospin, Karlsruhe, Germany) equipped with a 60-slot BACS sample changer using a QCI cryoprobe equipped with a cooled $^{31}$P coil and preamp and a one-axis self-shielded gradients. The samples were inserted sequentially and 10 minutes were given for temperature equilibration. Temperatures had been calibrated using d$_4$-methanol. [1] Topspin 3.2 was used to control the spectrometer. One-dimensional (1D) $^{31}$P experiments were recorded using 64k complex points with an acquisition time $t_{P,\text{max}} = 2.62$ sec (i.e. a spectral window of 25,000 Hz was used), a relaxation delay of 2.5 sec and 256 scans. Two-dimensional (2D) $^1$H,$^{31}$P-HMBC experiments[2, 3] were performed using a gradient version of the experiment in which a WATERGATE[4] water suppression had been implemented. 2,048 ($^1$H) $\cdot$ 64 ($^{31}$P) complex points were acquired, with acquisition times $t_{R,\text{max}} = 204.8$ ms and $t_{P,\text{max}} = 6.4$ ms (i.e. a spectral window of 10,000 Hz was used in each dimension) and 16 scans. NMR data were processed and spectra viewed using tospin 3.2 (Bruker Biospin).

**2.7 Photodeprotection**

UV-irradiation was carried out with a Hg (Xe) arc lamp (LOT-Quantum Design GmbH, Darmstadt, Germany) using a 297 nm filter with 15% transmission (Andover Inc., Salem, New Haven, USA). Samples were dissolved in MeOH at a concentration of 7.5 mM, positioned in 20 cm distance to the source and irradiated while stirring. The deprotection progress was followed by UPLC analysis. Upon complete conversion, a 10-fold excess of deep-frozen Et$_2$O was added and the mixture let sit in the freezer for 10 min. After centrifugation, the liquid phase was discarded and the precipitate re-suspended in the same amount of deep-frozen Et$_2$O. The mixture was kept in the freezer again for 10 min, centrifuged, decanted and the precipitate dried under reduced pressure for 15 min. Deprotected pLys substrates were stored in the freezer until applied in the assay.
2.8 Phosphatase Activity Assay

Phosphatase activities were determined on a SAFIRE\textsuperscript{2} microplate reader (TecanGroup Ltd., Männedorf, Switzerland) by photometric detection at 360 nm of released inorganic phosphate with the EnzCheck\textsuperscript{TM} Phosphatase Assay Kit (ThermoFisher Scientific\textsuperscript{TM}, Waltham, Massachusetts, USA) following the protocol for enzymatic kinetics.\cite{5} Briefly, substrates were incubated at a concentration of 100 μM in 50 mM Tris-HCl buffer containing 1 mM MgCl\textsubscript{2} and 1 mM ZnCl\textsubscript{2} at pH 7.8 in the presence or absence of 0.05 U ALP for the overall phosphate release (enzymatic plus background reaction, [E+BG]\textsubscript{wBL}) or the non-enzymatic hydrolysis ([BG]\textsubscript{wBL}), respectively.

The absorbance values of microplate, buffer, EnzCheck\textsuperscript{TM} reagent and ALP were determined in separated wells without adding substrate to the solution, considered as baseline ([BL]\textsubscript{w/ALP} and [BL]\textsubscript{w/oALP}). Reactions were run for 90 min in total, UV-absorbance was measured every 10 min. The enzymatic hydrolysis yield [E] was determined by subtraction of [BG]\textsubscript{w/oBL} from [E+BG]\textsubscript{w/oBL}.

\[
[E] = [E + BG]_{w/oBL} - [BG]_{w/oBL} = ([E + BG]_{w/BL} - [BL]_{w/ALP}) - ([BG]_{w/BL} - [BL]_{w/oALP})
\]

2.9 UV/Vis Spectroscopy

UV/Vis spectra and absorbance values were determined either on a V-630 spectrophotometer at r.t. or a V-550 UV/Vis spectrophotometer equipped with an ETC-505T temperature controller at 20 °C (both Jasco, Tokyo, Japan).

**Determination of resin loading for peptide synthesis**

1-2 mg dried resin were weighed into a microcentrifuge tube covered with 1 mL Fmoc deprotection solution (Pip:DMF, 1:4, v/v). After 10 min of shaking at r.t., the loading was determined in a 1 mL quartz cuvette. An aliquot of the deprotection solution was diluted to such an extent that the expected resulting absorbance was approx. 0.3. From the measured absorbance the loading was calculated by the help of the following equations:

\[
A_l = \varepsilon_\lambda \cdot c \cdot d
\]
$A$: Absorbance at given wavelength, $\varepsilon$: molar attenuation coefficient at given wavelength in $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ ($\varepsilon_{301\text{nm}} = 7800$ $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for 1-((9H-fluoren-9-yl)methyl)piperidine), $c$: concentration in mol$\cdot$L$^{-1}$, $d$: cuvette length in cm.

$$L_{\text{resin}} = \frac{A \times V_{\text{deprotection}} \times V_{cuvette}}{\varepsilon_{301\text{nm}} \times d \times V_{\text{valiquot}} \times m_{\text{resin}}}$$

$L_{\text{resin}}$: loading in $\mu\text{mol} \cdot \text{mg}^{-1}$; $A$: Absorbance at 301 nm; $V_{\text{deprotection}}$: volume of Fmoc deprotection solution in $\mu$L (1000 µL); $V_{cuvette}$: total volume in cuvette for measuring in µL; $\varepsilon_{301\text{nm}} = 7800$ $\mu\text{L} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$; $d$: cuvette length in cm; $m_{\text{resin}}$ : weighed amount in mg.

### 2.10 Electrostatic Potential Maps

Electrostatic potential (ESP) maps were calculated relying on density functional theory (DFT) optimized molecular structures. The exchange correlation functional B3LYP$^{[6-8]}$ together with the polarized triple zeta basis set def2-TZVPP$^{[9, 10]}$ on all atoms was employed and subsequent harmonic vibrational frequency analysis was carried out in order to check if a real local minimum on the potential energy surface was found. From the created electron densities, the electrostatic potential energy (in a.u.) was calculated and mapped on the respective density plot for a contour value of 0.01. The Turbomole program package V7.0.2$^{[11-13]}$ was used for all DFT electronic structure and geometry optimizations. ESP evaluations and visualizations were done with Molden 5.9.$^{[14, 15]}$
3. Organic Synthesis

3.1 Synthesis of homocysteine-derived phosphonate 1

3.1.1 Synthesis overview

Vinylphosphonic dichloride (crude)

Vinylphosphonic dichloride was synthesized as described before with minor adjustments. Briefly, in a heated Schlenk flask under Ar, 19 mL of dry DCM were cooled in an ice bath for 5 min. 1.34 mL (17.3 mmol) vinylphosphonic acid were added and cooled for 5 min before 5 drops of dry DMF were added. Under vigorous stirring, 4.47 mL (52.1 mmol, 3 eq.) oxalyl dichloride were added dropwise and the resulting orange mixture kept in the ice bath for further 15 min and then at rt. ovn. After 17.5 h solvents and oxalyl dichloride were evaporated under reduced pressure. The crude product was obtained as an orange-brown oil and used for the next step without further purification. $^1$H NMR (300 MHz, CDCl$_3$) δ 6.65–6.61 (m, 1H), 6.52–6.21 (m, 2H). $^{31}$P NMR (122 MHz, CDCl$_3$) δ 31.31.
3.1.3 Dibenzyl vinylphosphonate (4)

In a heated flask and in Ar atmosphere, 50 mL dry THF were pre-cooled. Subsequently, 4.5 mL (43.25 mmol, 2.5 eq.) benzyl alcohol and 7.2 mL (52.1 mmol, 3 eq.) Et$_3$N were added and cooled as well. 17.3 mmol (1 eq.) vinylphosphonic dichloride were dissolved in 10mL dry THF and added dropwise to the pre-cooled mixture, upon which a white precipitate was formed. The reaction was stirred for 15 min in ice bath and then at r.t. Next day, the precipitate was filtered off, solvents evaporated under reduced pressure and the crude product purified by silica column chromatography (100% DCM → DCM/EE 8/2, product eluting at 10% EE). The product was obtained as orange oil (3.24 g, 11.4 mmol, 66%). $^1$H NMR (600 MHz, CD$_3$CN) $\delta$ 7.43 – 7.31 (m, 10H), 6.29 – 6.05 (m, 3H), 5.07 – 4.96 (m, 4H). $^{31}$P NMR (243 MHz, CD$_3$CN) $\delta$ 14.70, 14.67. $^{13}$C NMR (151 MHz, CD$_3$CN) $\delta$ 139.44, 139.30, 138.27, 138.22, 137.19, 137.13, 131.83, 131.79, 131.58, 131.53, 131.18, 131.15, 131.12, 130.76, 130.73, 130.56, 130.54, 130.51, 130.48, 130.45, 130.12, 130.09, 130.06, 129.79, 128.69, 128.58, 127.50, 70.76, 69.80, 69.77, 68.79. Rf (DCM/EE 7/3, KMnO$_4$ stain) = 0.50. HR-MS for C$_{16}$H$_{17}$O$_3$P: m/z calc. [M+H$^+$]$^+$ = 289.0988, m/z obs. [M+H$^+$]$^+$ = 289.0994.

3.1.4 \(((9H-fluoren-9-yl)methoxy)carbonyl)-L-homocysteine (5)

At 0 °C, 480 mg (0.8 mmol) Fmoc-hCys(Trt)-OH were dissolved in 2.4 mL DCM and 2.6 mL of a mixture of TFA: TIS: EDT (96:3.8:0.2, v/v/v) added. Within 1 min the mixture turned yellow and back to colourless. UPLC analysis after 7 min indicated full conversion. Solvents were evaporated with nitrogen flush, while keeping the mixture in ice bath. Immediate purification on preparative HPLC gave 240 mg (0.67 mmol, 84%) of desired compound as white powder. $^1$H NMR (600 MHz, CD$_3$CN) $\delta$ 7.92 (d, $J = 7.5$ Hz, 2H), 7.76 (t, $J = 7.0$ Hz, 2H), 7.51 (t, $J = 7.5$ Hz, 2H), 7.35 (d, $J = 7.5$ Hz, 2H), 7.15 – 7.05 (m, 10H), 6.29 – 6.05 (m, 3H), 4.89 – 4.76 (m, 2H). $^{13}$C NMR (151 MHz, CD$_3$CN) $\delta$ 175.60, 150.10, 139.34, 138.09, 137.19, 137.13, 131.81, 131.77, 131.58, 131.53, 131.18, 131.15, 131.12, 130.78, 130.75, 130.56, 130.54, 130.51, 130.48, 130.45, 130.12, 130.09, 130.06, 129.79, 128.69, 128.58, 127.50, 70.76, 69.80, 69.77, 68.79. Rf (DCM/EE 7/3, KMnO$_4$ stain) = 0.30. HR-MS for C$_{22}$H$_{23}$NO$_4$: m/z calc. [M+H$^+$]$^+$ = 355.1506, m/z obs. [M+H$^+$]$^+$ = 355.1500.
Hz, 2H), 7.43 (tt, J = 7.5, 1.5 Hz, 2H), 6.07 (d, J = 8.4 Hz, 1H), 4.44 (d, J = 7.2 Hz, 2H), 4.39 (td, J = 8.8, 4.5 Hz, 1H), 4.33 (t, J = 7.0 Hz, 1H), 2.71 – 2.57 (m, 2H), 2.16 – 2.03 (m, 2H). \(^{13}\)C NMR (151MHz, CD\(_3\)CN) δ 175.35, 158.97, 146.81, 143.89, 131.00, 130.95, 130.40, 130.35, 129.94, 129.89, 129.33, 128.46, 127.38, 123.27, 123.22, 122.22, 122.17, 69.08, 55.53, 54.59, 50.22, 49.36, 39.08, 38.21, 37.36, 24.21, 23.25. HR-MS for C\(_{19}\)H\(_{19}\)NO\(_4\)S: m/z calc. [M+H\(^+\)]\(^+\) = 358.1108, m/z obs. [M+H\(^+\)]\(^+\) = 358.1119.

3.1.5 \(N-(((9H\text{-fluoren}-9-yl)methoxy)carbonyl)-S-(2-(bis(benzyloxy)phosphoryl)ethyl)-L\)-homocysteine, Fmoc-hCys(EtPO(OBn)\(_2\))-OH (1)

Thiol addition of Fmoc-hCys-OH 5 with alkene 4 was performed at basic pH as followed. 100 mg (280 µmol) AA and 242 mg (840 µmol, 3 eq.) alkene were dissolved in 1.5 mL DMF. After addition of 5 mL 50 mM AmBic buffer, pH 8.5 and pH adjustment to 8.7, the formed precipitate was redissolved with 1.2 mL additional DMF (final concentration of AA 45 mM). The reaction was stirred at 45 °C for 6 h until no starting material could be detected by UPLC analysis anymore. Solvents were evaporated under reduced pressure and by lyophilization and the crude mixture purified by preparative HPLC. 122.9 mg (190.4 µmol, 68%) of desired product were obtained as white powder. \(^1\)H NMR (600 MHz, CD\(_3\)CN) δ 7.81 (d, J = 7.5 Hz, 2H), 7.65 – 7.26 (m, 14H), 6.17 (d, J = 8.4 Hz, 1H), 5.10 – 4.92 (m, 4H), 4.31 (d, J = 6.9 Hz, 2H), 4.29 – 4.24 (m, 1H), 4.20 (t, J = 7.0 Hz, 1H), 2.71 – 2.58 (m, 2H), 2.59 – 2.50 (m, 2H), 2.19 – 1.83 (m, 4H). \(^{31}\)P NMR (243 MHz, CD\(_3\)CN) δ 30.15. \(^{13}\)C NMR (151 MHz, CD\(_3\)CN) δ 173.07, 156.28, 144.11, 144.01, 141.13, 136.53, 136.45, 128.61, 128.44, 127.99, 127.73, 127.14, 125.24, 120.00, 67.46, 67.44, 67.37, 67.35, 66.35, 52.79, 47.02, 31.04, 27.44, 27.18, 25.40, 23.88, 23.83. HR-MS for C\(_{35}\)H\(_{36}\)NO\(_7\)PS: m/z calc. [M+H\(^+\)]\(^+\) = 646.2043, m/z obs. [M+H\(^+\)]\(^+\) = 646.2042.
3.2 Synthesis of norleucine-derived phosphate 2

3.2.1 Synthesis overview

3.2.2 (2S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(((benzyloxy)(hydroxy)phosphoryl)oxy)hexanoic acid, Fmoc-Nle(OPO(OH)(OBn))-OH (2)

Fmoc-Nle(OPO(OH)(OBn))-OH 2 was prepared as previously described for the pSer derivative. Briefly, in a heated flask and under Ar, 16.5 mL dry THF were cooled in an ice bath for 5 min. 201 μL (2.29 mmol, 1.7 eq) phosphorous trichloride were added and cooled for 2 min as well. While cooling, 238 μL (2.29 mmol, 1.7 eq.) benzyl alcohol were added dropwise and the resulting solution kept at 0 °C for 5 min. Since $^{31}$P NMR indicated some PCl$_3$ left, further 0.3 eq. (0.4 mmol, 42 μL) benzyl alcohol were added and the mixture stirred for 5 min at 0 °C. Upon the addition of 490 μL (4.23 mmol, 3.1 eq.) 2,6-lutidine, a white precipitate was formed, to which a mixture of 500 mg (1.35 mmol, 1 eq.) Fmoc-Nle(6-OH)-OH, 2.8 mL dry THF and 167 μL (1.44 mmol, 1.07 eq.) 2,6-lutidine was dropped within 5 min. The flask was washed
with 0.6 mL dry THF to give a final concentration of AA of 65 mM. The reaction was kept for 10 min cooled and then stirred at r.t. until UPLC analysis indicated full consumption of the AA (90 min). Successively, 1.7 mL H2O, 321 mg (3.12 mmol, 2.3 eq) sodium bromide and 510 µL of a 20% sodium bromate solution (containing 102 mg, 0.7 mmol, 0.51 eq NaBrO₃) were added, upon which the mixture turned orange. After 5 min cooling with ice, the reaction was stirred at r.t. for 4 h, when UPLC analysis indicated formation of the desired product. Remaining oxidation reagent was quenched with 10 mL of a Na₂S₂O₅ solution as could be observed by destaining of the mixture. The crude product was obtained after evaporation of the solvents under reduced pressure and with lyophilization. 561 mg (1.04 mmol, 77%) of monobenzyl/Fmoc protected phospho-AA were obtained after purification using preparative HPLC. ¹H NMR (600 MHz, CD₃CN) δ 7.81 (d, J = 7.6 Hz, 2H), 7.65 (t, J = 6.7 Hz, 2H), 7.41 – 7.37 (m, 2H), 7.37 – 7.34 (m, 5H), 7.33 – 7.29 (m, 2H), 6.05 (s, 1H), 5.02 (dd, J = 8.1, 2.2 Hz, 2H), 4.33 – 4.28 (m, 2H), 4.20 (t, J = 7.0 Hz, 1H), 4.12 (d, J = 7.5 Hz, 1H), 3.98 (q, J = 6.6 Hz, 2H), 1.84 – 1.55 (m, 4H), 1.41 (dq, J = 15.7, 7.4 Hz, 2H). ³¹P NMR (243 MHz, CD₃CN) δ 3.40. ¹³C NMR (151 MHz, CD₃CN) δ 176.05, 160.46, 160.19, 159.00, 146.78, 143.85, 138.96, 131.84, 131.79, 131.69, 131.13, 130.99, 130.94, 130.77, 130.73, 130.62, 130.39, 130.35, 130.06, 129.93, 129.88, 129.33, 129.29, 128.45, 127.40, 123.26, 123.20, 122.20, 122.15, 71.61, 70.62, 70.06, 69.10, 56.90, 55.98, 50.17, 49.30, 32.03, 24.21. HR-MS for C₂₈H₃₀NO₈P: m/z calc [M+H⁺]⁺ = 540.1782, m/z obs. [M+H⁺]⁺ = 540.1784.

3.3 Peptide synthesis with building blocks 1 and 2

3.3.1 Synthesis of AcTyr-hCys(EtPO(OH)₂)-Gly²⁻NH₂ (8a)
Peptide 8a was synthesized as described in section 2.2 on a 50 µmol scale with resin 17. Incorporation of building block 1 was conducted as followed: 2 eq. AA (100 µmol, 65 mg) and 1.95 eq. HATU (97.5 µmol, 37.1 mg) were dissolved in 500 µL DMF to give a 0.2 M solution. Directly before adding the mixture to the Fmoc-deprotected resin, 34 µL (4 eq., 200 µmol) DIPEA were added. The mixture was shaken at r.t. for 2 h, when the TNBS test indicated full conversion. The peptide was capped before continuation of the peptide coupling. The crude product was purified on an analytical HPLC column to give 11.7 mg (23.5 µmol, 47% with regard to initial resin loading) white powder of desired peptide 8a.

$^1$H NMR (600 MHz, H$_2$O+10% D$_2$O, pH 2) $\delta$ 8.27 (d, $J = 7.5$ Hz, 1H), 8.13 (d, $J = 5.8$ Hz, 1H), 7.31 (t, $J = 6.2$ Hz, 1H), 7.18 (s, 1H), 6.89 (s, 1H), 6.83 (d, $J = 8.5$ Hz, 2H), 6.51 (d, $J = 8.5$ Hz, 2H), 4.10 (m, 1H), 3.58 – 3.39 (m, 2H), 2.65 (ddd, $J = 70.4, 13.8, 7.9$ Hz, 2H), 2.40 – 2.32 (m, 2H), 2.14 (ddt, $J = 96.9, 14.6, 7.8$ Hz, 2H), 1.85 (s, 1H), 1.66 (s, 3H), 1.64 (s, 1H), 1.57 (ddt, $J = 18.4, 8.9, 3.8$ Hz, 3H). $^{31}$P NMR (243 MHz, H$_2$O+10% D$_2$O, pH 2) $\delta$ 23.13. $^{13}$C NMR (600 MHz, D$_2$O) $\delta$ 176.90, 176.54, 176.50, 176.04, 157.29, 133.31, 130.46, 118.24, 118.19, 58.37, 55.18, 44.83, 38.69, 32.70, 30.83, 29.96, 29.67, 27.27, 27.25, 24.26. HR-MS for C$_{19}$H$_{25}$N$_4$O$_8$PS: $m/z$ calc. [M+H$^+$]+ = 505.1517, $m/z$ obs. [M+H$^+$]+ = 505.1521. $t_R$ (gradient II) = 3.691 min.

### 3.3.2 Synthesis of AcTyr-Nle(OPO(OH)$_2$)-Gly$^{CONH_2}$ (8b)

Peptide 8b was synthesized as described in section 2.2 on a 50 µmol scale with resin 17. Incorporation of building block 2 was conducted as followed: 2 eq. AA (100 µmol, 54 mg) and 1.95 eq. HATU (97.5 µmol, 37.1 mg) were dissolved in 500 µL DMF to give a 0.2 M solution. Directly before adding the mixture to the Fmoc-deprotected resin, 34 µL (4 eq., 200 µmol) DIPEA were added. The mixture was shaken at r.t. for 2 h, when the TNBS test indicated full conversion. The peptide was capped before continuation of the peptide coupling. The crude product was purified on an analytical HPLC column to give 19.3 mg (39.5 µmol, 79% with regard to initial resin loading) white powder of desired peptide 8b. $^1$H NMR (600 MHz, D$_2$O) $\delta$ 7.13 – 7.07 (m, 2H),
3.4 Fmoc-based synthesis of caged phospho-lysine peptide

3.4.1 Synthesis of the Tc-protected building block 3

3.4.1.1 Synthesis overview

3.4.1.2 Benzyl ((benzyloxy)carbonyl)-L-lysinate, Z-Lys-OBn (10)
Benzyl protection of ((benzyloxy)carbonyl)-L-lysine was achieved by nucleophilic substitution. 1g (3.6 mmol) Z-Lys-OH was dissolved in benzyl alcohol to a 0.8 M solution and cooled in an ice bath for 5 min. 1.4 mL (18 mmol, 5 eq.) thionyl chloride were added dropwise to the mixture. After complete addition, the reaction was stirred 2 h at r.t. until UPLC analysis indicated complete conversion. The mixture was concentrated under reduced pressure and purified via silica column chromatography (100% EE+1% Et₃N → MeOH/EE 25/25+1% Et₃N, product eluting at 25% MeOH, silica filtered off after solvent evaporation). 1.20 g (3.2 mmol, 90%) product were obtained as colorless oil. 

$^1$H NMR (600 MHz, CD$_3$CN) δ 7.43 – 7.25 (m, 10H), 6.27 (d, J = 8.1 Hz, 1H), 5.15 (q, J = 12.7 Hz, 2H), 5.08 (q, J = 12.6 Hz, 2H), 4.22 (td, J = 8.6, 4.9 Hz, 1H), 2.88 (t, J = 7.7 Hz, 2H), 1.85 – 1.65 (m, 2H), 1.63 (ddd, J = 21.0, 11.5, 4.3 Hz, 2H), 1.40 (qd, J = 9.2, 8.1, 5.1 Hz, 2H). 

$^{13}$C NMR (151 MHz, CD$_3$CN) δ 175.01, 163.84, 163.61, 163.39, 163.16, 159.17, 158.56, 139.78, 138.81, 131.84, 131.80, 131.74, 131.55, 131.50, 131.46, 131.34, 131.29, 131.25, 131.04, 131.01, 130.95, 130.78, 130.73, 130.68, 130.44, 130.24, 130.17, 129.95, 129.92, 129.88, 122.56, 120.61, 118.67, 116.72, 70.32, 69.98, 69.32, 69.00, 68.33, 68.02, 57.35, 56.41, 43.02, 42.07, 41.12, 34.22, 33.36, 32.51, 30.06, 29.22, 28.37, 25.82, 24.99, 24.14. 

RF (EE/MeOH 3/1+1% Et₃N) = 0.10. HR-MS for C$_{21}$H$_{26}$N$_2$O$_4$: m/z calc. [M+H$^+$]$^+$ = 371.1966, m/z obs. [M+H$^+$]$^+$ = 371.1971

3.4.1.3 Benzyl N2-((benzyloxy)carbonyl)-N6-(bis(2,2,2-trichloroethoxy)phosphoryl)-L-lysinate, Z-Lys(NPO(OTc)$_2$)-OBn (11)

![Chemical structure of Compound 11](image)

Compound 11 was synthesized in accordance to the previously described protocol by the Seebeck group.$^{[17]}$ Briefly, 1.20 g (3.24 mmol) 10 were dissolved to a 60 mM solution in ACN (54 mL) and 4 eq. Et₃N (13 mmol, 1.8 mL) were added and the pH checked for basicity. 1.47 g (3.88 mmol, 1.2 eq.) bis(2,2,2-trichloroethyl) phosphorochloridate were added in three equal portions of 491 mg each every 2 h while stirring at r.t. The reaction was kept at r.t. oven and TLC indicated full conversion. Solvents were evaporated under reduced pressure and the crude product purified via silica column (hex/EE 8/2+0.25% FA → hex/EE 3/7+0.25% FA, product eluting at 40-
50% EE). Fractions containing 11 were concentrated under reduced pressure and residue formic acid removed by lyophilization. The product was obtained as a colourless oil (1.71 g, 2.40 mmol, 74%). $^1$H NMR (600 MHz, CD$_3$CN) $\delta$ 7.43 – 7.26 (m, 10H), 6.08 (d, $J$ = 8.1 Hz, 1H), 5.13 (q, $J$ = 9.3 Hz, 2H), 5.07 (q, $J$ = 8.1 Hz, 2H), 4.59 (dd, $J$ = 6.2, 1.4 Hz, 4H), 4.24 – 4.15 (m, 1H), 3.92 (dt, $J$ = 13.4, 6.9 Hz, 1H), 2.95 (dddd, $J$ = 13.8, 11.8, 6.8, 1.2 Hz, 2H), 1.83 – 1.61 (m, 2H), 1.58 – 1.44 (m, 2H), 1.43 – 1.36 (m, 2H). $^{31}$P NMR (243 MHz, CD$_3$CN) $\delta$ 4.52, 4.50, 4.48, 4.45, 4.43. $^{13}$C NMR (151 MHz, CD$_3$CN) $\delta$ 175.07, 158.98, 139.85, 138.86, 131.82, 131.77, 131.72, 131.47, 131.27, 131.20, 130.95, 130.76, 130.71, 130.65, 130.40, 130.21, 129.90, 98.14, 98.06, 79.79, 79.76, 78.75, 78.73, 77.72, 77.69, 70.17, 69.85, 69.19, 68.87, 68.20, 67.89, 57.43, 56.49, 44.39, 43.47, 42.55, 34.43, 34.12, 33.55, 33.28, 32.71, 32.46, 25.84, 25.03, 24.20. $R_f$ (hex/EE 7/3+0.25% FA) = 0.37. HR-MS for C$_{25}$H$_{29}$Cl$_6$N$_2$O$_7$P: $m/z$ calc. [M+H$^+$$]^+$ = 710.9917, $m/z$ obs. [M+H$^+$$]^+$ = 710.9915.

3.4.1.4 N6-(bis(2,2,2-trichloroethoxy)phosphoryl)-L-lysine, H$_2$N-Lys(NPO(OTc)$_2$)-OH

$N$- and C-terminal deprotection was achieved with hydrogenation. 600 mg (850 µmol) 11 were weighed into a Schlenk flask, dissolved to a 75 mM solution with AcOH:TFA:MeOH (5:5:90, v/v/v, 11 mL) and kept under vacuum for 5 min. In inert atmosphere, 96 mg Pd on activated charcoal (10% Pd, 113 mg per mmol starting material) were added and the gas exchanged with H$_2$. The mixture was stirred for 45 min in H$_2$ atmosphere until UPLC analysis indicated full conversion. The catalyst was filtered off and washed with MeOH. Solvents were evaporated by bubbling N$_2$ through the solution and residual acid removed by lyophilization. The crude product was applied in the next step without further purification.
3.4.1.5  *N*₂-(((9*H*-fluoren-9-*y*l)methoxy)carbonyl)-*N*₆-(bis(2,2,2-trichloroethoxy)phosphoryl)-*L*-lysine, Fmoc-*L*ys(NPO(OTc)₂)-OH (3)

850 μmol crude product of H₂N-Lys(NPO(OTc)₂)-OH were dissolved in 2.1 mL H₂O to a 0.4 M solution and 120 μL (850 μmol, 1 eq.) Et₃N were added. The pH was adjusted to 9 and a 0.4 M solution of 287 mg (850 μmol, 1 eq.) Fmoc-OSu in ACN (2.1 mL) was added while keeping the pH above 9 by further Et₃N addition. The reaction was allowed to proceed until the pH stabilized at pH 9.2. Subsequently, the pH was adjusted to 3.5 with AcOH and the mixture extracted 3x with CHCl₃. The combined organic layers were dried over Na₂SO₄ and solvents evaporated under reduced pressure. The crude product was purified via flash silica column (1. wash impurities off with hex/EE 45/55+0.25% FA, 2. elution of product with hex/EE 35/65+0.25% FA). After evaporation and lyophilization, 357 mg (502 μmol, 59%) product were obtained as white powder. ¹H NMR (600 MHz, CD₃CN) δ 7.83 (d, *J* = 7.7 Hz, 2H), 7.68 (t, *J* = 6.6 Hz, 2H), 7.42 (t, *J* = 7.6 Hz, 2H), 7.36−7.31 (m, 2H), 6.07 (d, *J* = 8.0 Hz, 1H), 4.60 (d, *J* = 6.2 Hz, 4H), 4.33 (d, *J* = 7.2 Hz, 2H), 4.23 (t, *J* = 7.0 Hz, 1H), 4.13−4.05 (m, 1H), 3.94 (dt, *J* = 13.4, 6.9 Hz, 1H), 2.99 (dq, *J* = 13.4, 6.9 Hz, 2H), 1.84−1.62 (m, 1H), 1.59−1.47 (m, 2H), 1.43 (q, *J* = 8.2, 7.3 Hz, 2H). ³¹P NMR (243 MHz, CD₃CN) δ 4.59, 4.57, 4.54, 4.52, 4.49, 4.46, 4.44, 4.41, 4.39. ¹³C NMR (151 MHz, CD₃CN) δ 176.08, 158.93, 146.86, 143.86, 130.95, 130.35, 129.89, 129.30, 123.27, 123.21, 122.15, 98.11, 79.77, 78.73, 77.70, 68.97, 57.03, 56.11, 50.21, 49.49, 49.34. Rf (hex/EE: 1/1+0.25% FA) = 0.47. HR-MS for C₂₅H₂₇Cl₆N₂O₇P: *m/z* calc. [M+H⁺]+ = 708.9760, *m/z* obs. [M+H⁺]+ = 708.9764.
3.4.2 Peptide synthesis with building block 3 (peptide 12)

Peptide 12 was synthesized as described in section 2.2 on a 20 µmol scale with resin 17. Incorporation of building block 3 was conducted as followed: 2.1 eq. AA (42 µmol, 30 mg) and 2 eq. HATU (40 µmol, 15 mg) were dissolved in 200 µL DMF to give a 0.2 M solution. Directly before adding the mixture to the Fmoc deprotected resin, 14 µL (4 eq., 80 µmol) DIPEA were added. The mixture was shaken at r.t. for 2 h, when the 2,4,6-trinitrobenzene-sulphonic acid (TNBS) test indicated full conversion. The peptide was capped before continuation of the peptide coupling. The crude product was purified on an analytical HPLC column to give 2.1 mg (2.8 µmol, 14% with regard to initial resin loading) white powder of desired peptide 12. $^1$H NMR (600 MHz, CD$_3$CN+10% D$_2$O) $\delta$ 7.14 (d, $J = 8.5$ Hz, 2H), 6.80 (d, $J = 8.5$ Hz, 2H), 4.69 (dd, $J = 6.1$, 1.7 Hz, 4H), 4.53 – 4.47 (m, 1H), 4.19 (dd, $J = 9.2$, 4.8 Hz, 1H), 3.78 (dd, $J = 25.2$, 17.0 Hz, 2H), 2.90 (dd, $J = 14.0$, 8.1 Hz, 2H), 1.95 (s, 3H), 1.88 – 1.81 (m, 2H), 1.66 (dtd, $J = 14.2$, 9.7, 5.1 Hz, 2H), 1.57 (ddt, $J = 17.7$, 9.1, 6.8 Hz, 2H), 1.40 – 1.32 (m, 2H). $^{31}$P NMR (243MHz, CD$_3$CN+10% D$_2$O) $\delta$ 4.83, 4.81, 4.79, 4.76, 4.74, 4.71, 4.69. $^{13}$C NMR (151 MHz, CD$_3$CN) $\delta$ 175.24, 174.98, 174.85, 174.24, 158.50, 133.13, 130.46, 117.84, 97.89, 78.80, 58.04, 56.40, 44.68, 43.38, 38.92, 33.28, 32.99, 25.03, 24.57. HR-MS for C$_{23}$H$_{32}$Cl$_6$N$_5$O$_8$P: $m/z$ calc. [M+H]$^+$ = 748.0193, $m/z$ obs. [M+H]$^+$ = 748.0192. $t_R$ (gradient) = 6.179 min.
3.5 Synthesis of phospho-lysine peptide 8c via the Staudinger-phosphite reaction

3.5.1 Synthesis overview

![Synthesis Diagram]

3.5.2 1-(2-nitrophenyl)ethanol (14)

In a 500 mL round-bottom flask 10 g (60.6 mmol) 2'-nitroacetophenone were dissolved in 100 mL MeOH:dioxane (3:2, v/v) and cooled with an ice bath. Under vigorous stirring, 2.5 eq. sodium borohydride (151.4 mmol, 5.7 g) were added portion wise over 90 min. The resulting mixture was equipped with septum and balloon and left to warm to r.t. oven while stirring. After 16 h residual NaBH₄ was quenched by the addition of 50 mL of acetone and the solvents evaporated under reduced pressure. The residue was diluted with H₂O and EE and the layers separated. The organic layer was washed twice with water, the combined aqueous layers were washed once with EE, eventually, the combined organic layers were washed with brine, dried over MgSO₄ and the filtered solution concentrated under reduced pressure. The residual solvent was evaporated under high vacuum oven. The product (9.7 g, 58.2 mmol, 96%) was obtained as a yellow oil. ¹H-NMR (600 MHz, CD₃CN) δ 7.86 (dd, J = 8.1, 1.4 Hz, 2H), 7.71 (td, J = 7.6, 1.3 Hz, 1H), 7.47 (ddd, J = 8.7, 7.4, 1.5 Hz, 1H), 5.30 (qd, J = 6.4, 4.1 Hz, 1H), 3.55 (dd, J = 4.2, 1.3 Hz, 1H), 1.48 (d, J = 6.4 Hz, 3H). ¹³C-NMR (151 MHz, CD₃CN) δ 147.84, 141.58, 133.31, 127.91, 127.62, 123.75, 64.64, 24.19. HR-MS for C₈H₆NO₃: m/z calc. [M+H⁺]+ = 166.0499, m/z obs. [M+H⁺]+ = 166.0505.
3.5.3 Tris(1-(2-nitrophenyl)ethyl) phosphite (15)

Phosphite 15 was synthesized by the condensation of trichlorophosphane and the alcohol 14 under inert conditions. A Schlenk flask was equipped with 1.7 g 1-(2 nitrophenyl)ethanol 14 (10.2 mmol, 3.4 eq.) and magnetic stir bar and set under high vacuum for 10 min. After balancing the pressure with argon, 10 mL dry THF were added and the solution cooled in an ice bath for 5 min. 1.33 mL Et$_3$N (9.6 mmol, 3.2 eq.) were added, the mixture left for another 2 min, while preparing a 0.6 M solution of 0.27 mL trichlorophosphane (3 mmol, 1.0 eq.) in dry THF (5 mL). The PCl$_3$ solution was added dropwise to the Schlenk flask, whereby a white precipitate was formed. After complete addition the mixture was kept in the ice bath for another 15 min, then stirred at r.t. under exclusion of light oven. After 18 h the precipitate was filtered, washed with EE and the filtrate concentrated under reduced pressure. 1.03 g (2.19 mmol, 73%) product were obtained after column chromatography (hex/EE 9/1+1% Et$_3$N → hex/EE 7/3+1% Et$_3$N, the product eluting at 25% EE) as yellow oil and kept under argon, protected from light in the freezer until further usage. $^1$H-NMR (300MHz, CD$_3$CN) δ 7.92 – 7.74 (m, 3H), 7.70 – 7.34 (m, 9H), 5.84 – 5.57 (m, 3H), 1.52 – 1.37 (m, 6H), 1.29 – 1.19 (m, 3H). $^{31}$P-NMR (122 MHz, CD$_3$CN) δ 137.82, 135.97. $^{13}$C-NMR (75 MHz, CD$_3$CN) δ 146.95, 146.86, 146.71, 146.44, 139.09, 139.04, 138.92, 138.89, 133.75, 133.73, 128.51, 128.42, 128.29, 128.02, 127.98, 127.90, 127.68, 124.10, 124.07, 124.06, 67.01, 66.83, 66.76, 66.64, 66.61, 66.50, 24.33, 24.28, 24.24, 24.20, 24.18, 24.13, 24.03, 23.98. Rf (hex/EE 3/1+1% Et$_3$N) = 0.57.
3.5.4 \text{AcTyr-Lys(N)}_3\text{-GlyCONH}_2 (16)

Peptide 16 was synthesized as described in section 2.2 on a 20 \mu mol scale with resin 17. Incorporation of Fmoc-az-Lys-OH was conducted as described in section 2.2 with HATU as activating reagent. The mixture was shaken at r.t. for 2 h, when the TNBS test indicated full conversion. The peptide was capped before continuation of the peptide coupling. The crude product was purified on an analytical HPLC column to give 5.11 mg (11.8 \mu mol, 59\% with regard to initial resin loading) white powder of desired peptide 16. HR-MS for C_{19}H_{27}N_7O_5: m/z calc. [M+H]^+ = 433.2074, m/z obs. [M+H]^+ = 433.2079. t_R (gradient II) = 3.458 min.

3.5.5 \text{AcTyr-Lys(NPO(ONPE))_2-GlyCONH}_2 (13)

5.1 mg (11.8 \mu mol) azido-peptide were dissolved in 400 \mu L dry DMF. 18.7 mg (35.4 \mu mol, 3 eq.) phosphite 15 were dissolved in 72 \mu L dry DMF and added to the azide (final azide concentration 25 mM). The reaction was kept at 45 °C and samples taken for UPLC analysis at distinct time points. After 7 h and 26 h additional 2 eq. phosphite 15 were added to the reaction in order to drive it to completion. After 48 h, solvents were evaporated under reduced pressure and residual DMF removed during lyophilization. The product was obtained as a white powder after purification via analytical HPLC (7.0 mg, 9.0 \mu mol, 76\%). HR-MS for C_{35}H_{44}N_7O_{12}P: m/z calc. [M+H]^+ = 786.2865, m/z obs. [M+H]^+ = 786.2870. t_R (gradient I) = 6.212 min.
0.5 µmol caged-pLys peptide 14 (0.39 mg) was deprotected and worked up as described in the photodeprotection section 2.7. HR-MS for C_{19}H_{30}N_{5}O_{8}P: m/z calc. [M+H]^+ = 488.1911, m/z obs. [M+H]^+ = 488.1923. \( t_R \) (gradient II) = 1.537 min.
4. UPLC/UV Chromatograms at 220 nm

Peptide \(^{\text{AcTyr-hCys(EtPO(OH)}_2\text{-Gly}}\)^{\text{CONH}_2} (8a)

Peptide \(^{\text{AcTyr-Nle(OPO(OH)}_2\text{-Gly}}\)^{\text{CONH}_2} (8b)
Peptide $\text{Ac}^{\text{Tyr-Lys(NPO(OTc)\text{2})}}\text{-Gly}^{\text{CONH2}}$ (12)

Peptide $\text{Ac}^{\text{Tyr-Lys(NPO(ONPE)\text{2})}}\text{-Gly}^{\text{CONH2}}$ (13)
Peptide $^{Ac}$Tyr-Lys(N$_3$)-Gly$^{CONH_2}$ (16)

Peptide $^{Ac}$Tyr-pLys-Gly$^{CONH_2}$ (8c)
5. NMR Spectra

Vinylphosphonic dichloride (crude)

Dibenzyl vinylphosphonate (4)
$^3$P NMR: (203 MHz, CD$_3$CN) δ 14.78, 14.67.

$^{13}$C NMR: (151 MHz, CD$_3$CN) δ 139.48, 139.30, 138.27, 138.22, 137.18, 137.13, 131.83, 131.79, 131.58, 131.53, 131.18, 131.14, 131.12, 130.75, 130.73, 130.56, 130.54, 130.51, 130.48, 130.45, 130.12, 130.09, 130.06, 129.79, 128.60, 128.58, 127.50, 127.48, 126.80, 60.77, 68.79.
(((9H-fluoren-9-yl)methoxy)carbonyl)-L-homocysteine (5)
$N$-((($9H$-fluoren$-9$-yl)methoxy)carbonyl)$-S$-(2-(bis(benzyloxy)phosphoryl)ethyl)-$L$-homocysteine (1)
$^1$H NMR (125 MHz, CD$_3$N): δ 8.15.

$^{13}$C NMR (75 MHz, CD$_3$N): δ 175.15, 156.28, 154.11, 154.06, 143.13, 136.53, 136.45, 128.64, 128.44, 127.99, 127.75, 127.14, 125.24, 120.00, 67.86, 67.84, 67.37, 67.35, 66.35, 52.79, 47.62, 31.04, 31.44, 27.18, 23.46, 23.36, 23.35.
(2S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(((benzyloxy)(hydroxy)phosphoryl)oxy) hexanoic acid (2)
AcTyr-Nle(OPO(OH)₂)-GlyCONH₂ (8b)
39
Benzyl ((benzyloxy)carbonyl)-L-lysinate (10)
Benzyl N2-((benzyl oxy)carbonyl)-N6-(bis(2,2,2-trichloroethoxy)phosphoryl)-L-lysinate (11)
$N_2-$(((9H-fluoren-9-yl)methoxy)carbonyl)-$N_6$-(bis(2,2,2-trichloroethoxy) phosphoryl)-L-lysine (3)
Ac-Tyr-Lys(NPO(OTc)2)-Gly\textsuperscript{CONH2} (12)
1-(2-nitrophenyl)ethanol (14)
Tris(1-(2-nitrophenyl)ethyl) phosphite (15)
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