Electronic Supporting Information

for

"Modular Solid-Phase Synthesis of Electrophilic Cysteine-Selective Ethynyl-Phosphonamidate Peptides"

1 General Information

1.1 General Description of Methods

Air or moisture sensitive reactions were carried out under argon atmosphere and at room temperature (20-25 °C). Column chromatography was performed using NORMASIL 60® silica gel 40-63 µm (VWR international, USA). Glass TLC plates, silica gel 60 W coated with fluorescent indicator F254s were purchased from Merck (Merck Group, Germany). Spots were visualized by manganese staining (10 g K_2CO_3, 1.5 g $KMnO_4$, 0.1 g NaOH in 200 ml H_2O) and heating. Chemicals and solvents were, unless stated otherwise, commercially available and used without further purification. The reagents were purchased from Sigma-Aldrich, Merck (Merck group Germany), TCI (Tokyo chemical industry) and Acros Organics (Thermo Fisher scientific, USA). Resins and Fmoc-protected amino acids were purchased from IRIS BioTech (Germany) or Novabiochem (Germany).

1.2 UPLC-ESI-MS

UPLC-UV/MS traces were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters autosampler, a Waters TUV detector and a Waters Acquity QDa detector with an Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min (Waters Corp., USA). The following gradient was used A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 5 → 95% B, 0-5 min. UV chromatograms were recorded at 220 nm. As some UV-signals overlapped, peak ratios were recorded by performing a QDa: MS scan (QDa positive scan 100.00 – 1250.00 Da, centroid CV=15) and relative peak area integration.

1.3 General Procedure for Test Cleavages of 3a

1 mg of resin was suspended in 300 µL of **cocktail I** (95% TFA, 2.5% TIS, 2.5% H_2O). The test cleavages were performed for 30, 60, 90 or 240 minutes at room temperature under slight agitation in the dark. Then, 400 µL of H_20/MeCN (50:50) were added and the suspension was filtered. For UPLC-ESI-MS analysis, the sample was diluted 1:3 with H_2O/MeCN (50:50) and the ratio of product to side products was determined by relative peak area integration.

1.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded with a Bruker Avance III 600 MHz spectrometer (Bruker Corp., USA) at ambient temperature. Chemical shifts δ are reported in ppm relative to residual solvent peaks.

1.5 Semi-Preparative HPLC

Semi-preparative HPLC was performed on a Shimadzu prominence HPLC system (Shimadzu Corp., Japan) with a CBM20A communication bus module, an FRC-10A fraction collector, 2 pumps LC-20AP, and an SPD-20A UV/VIS detector, using a VP250/10 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany). The following conditions were used: (A = H_2O + 0.1% TFA, B = MeCN + +0.1% TFA), flow rate 10 ml/min, 5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min.

1.6 High-Resolution Mass Spectrometry (HRMS)

High resolution ESI-MS spectra were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C18 column (1.7 µm, 2.1 mm x 50 mm). Samples were eluted with a flow rate of 0.3 mL/min. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. 5% B: 0-1 min; 5 to 95% B: 1-7min; 95% B: 7 to 8.5 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer.

1.7 Solid-Phase Peptide Synthesis (SPPS)

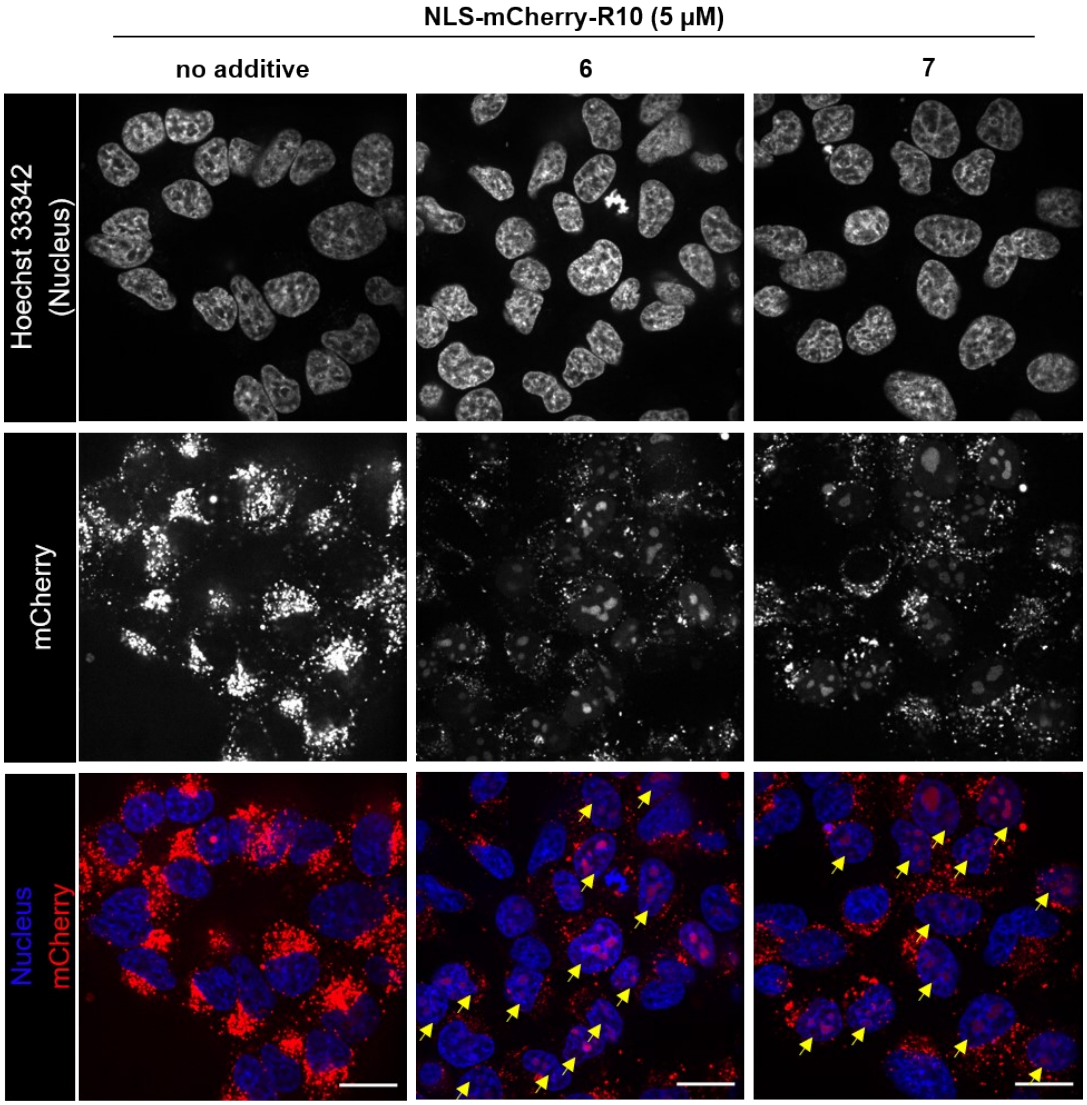
Model peptides were synthesized on a Tribute-UV automated PTI peptide synthesizer (Protein technologies, USA) via standard Fmoc-based protocols.

1.8 Cellular Uptake Experiment with Live HeLa CCl-2 Cells[1]

HeLa-CCL2 cells were maintained in high glucose Dulbecco's Minimum Eagle Medium (DMEM) supplemented with L-glutamine and 10% fetal calf serum (FCS) in a humidified 37°C with 5% CO2 incubator. Cells were passaged every 48 h.

HeLa-CCL2 cells (25000 cells) were seeded onto a black-walled glass bottom 96-well plate (CellVis) and were left to adhere for 48 h at 37°C in DMEM with 10% FCS. The cells were washed with phosphate buffered saline (PBS; 200 µL; 1×) and treated with 5 µM NLS-mCherry-R_{10} (synthesis found in reference

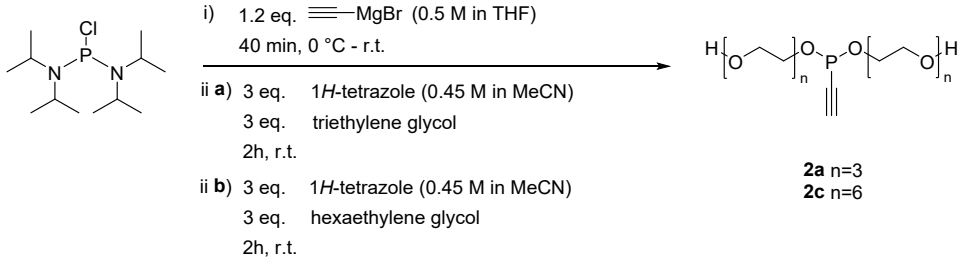
25) with or without 10 µM CPP-additive (indicated in Figure 2) in FluoroBrite DMEM (Gibco) at 37°C for 1 h. After washing the cells with without PBS (200 µL, 1×), treated cells were counterstained with Hoechst 33342 in FluoroBrite DMEM for 1 min, washed with PBS (200 µL, 1×). The media was changed to FluoroBrite DMEM with 10% FCS, followed by live-cell confocal microscopy using a Nikon-CSU spinning disc microscope with an CSU-X1 (Andor) and a live cell incubation chamber (OKOlab). All images shown in this work were acquired using a PlanApo 60x NA 1.4 oil objective (Nikon) and an EMCCD (AU888, Andor). Brightfield images were taken along with fluorescence images. Standard laser, a quad Dicroic (400-410,486-491, 560-570,633-647, AHF) and Emission filters were used in the acquisition of confocal fluorescence images; Hoechst 33342 (ex.: 405 nm em.: 450/50) and mCherry (ex.: 587 nm, em.: 410 nm) filter sets.



Supplementary Figure S1. HeLa-CCL2 cells treated with NLS-mCherry-R10 (5 µM) and indicated CPP-additives (10 µM) for 1 h. Wider field of image is presented to show more cells per image. Scale bar = 20 µm

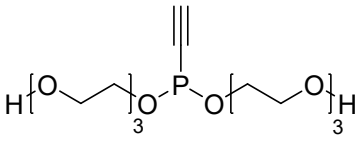
2 Synthesis

2.1 General Procedure I for the Synthesis of PEGylated Ethynyl-Phosphonites[2]



Under argon atmosphere, a 50-mL Schlenk flask was charged with 267 mg bis-(diisopropylamino)-chlorophosphine (1 mmol) and cooled to 0 °C. Subsequently, 2.40 mL ethynyl-magnesium-bromide solution (0.5 M in THF, 1.2 mmol, 1.2 eq.) was added dropwise. The suspension was allowed to warm up to room temperature and stirred for further 40 minutes. Subsequently, triethylene - or hexaethylene glycol (3 mmol, 3 eq.), dissolved in 6.67 mL 1*H*-tetrazole solution (0.45 M in MeCN, 3.00 mmol, 3 eq.), was added and the off- white suspension was stirred for 2 hours at room temperature. Under argon atmosphere, a 50 mL falcon tube was charged with the reaction mixture and centrifuged at 8000 rpm for 10 min. The supernatant was placed on a silica gel flash column for purification (0-5% MeOH in CH_2Cl_2). After purification, the solvents were evaporated *in vacuo* and the product was immediately resolubilized in 500 µL dry DMF. Next, 5 µL of the resulting solution were diluted with 495 µL dry DMF and phosphonoacetic acid (2 mg) was added as an internal standard. The concentration and yield of the product was determined by $^{31}P\{^1H\}$-NMR and relative peak area integration.

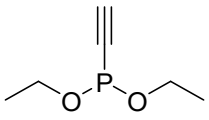
2.2 Synthesis of Ethynyl-Phosphonite **2a**



Ethynyl-phosphonite **2a** was synthesized according to General Procedure I from 267 mg bis-(diisopropylamino)-chlorophosphine (1 mmol, 1 eq.) and 0.30 mL triethylene glycol (3 mmol, 3 eq.). The desired compound was obtained as a yellow oil. (132.17 mg, 0.37 mmol, 37%). R_f = 0.3

$^{31}P\{^1H\}$-NMR (243 MHz, DMSO-d6) δ [ppm] = 130.5.

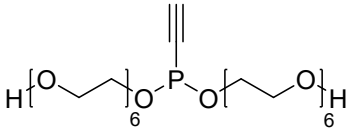
2.3 Synthesis of Ethynyl-Phosphonite 2b[3]



Under argon atmosphere, a 25 mL Schlenk-flask was charged with 2.00 mL ethynyl-magnesium-bromide solution (0.5 M in THF, 1.2 eq.). The solution was cooled to -78 °C and 143 µL diethyl-chloro-phosphite (1 mmol, 1 eq.) were added. The solution was stirred for 30 minutes at this temperature, then allowed to warm up to room temperature and stirred for further 60 minutes. The reaction solution was concentrated *in vacuo* and the crude product was purified by flash column chromatography (hexane/ethylacetate 92:8) to yield **2b** as a yellow oil (0.067 mmol, 6.7%)**.** After purification, the solvents were evaporated *in vacuo* and the product was immediately resolubilized in 500 µL dry DMF. Next, 5 µL of the resulting solution were diluted with 495 µL dry DMF and phosphonoacetic acid (2 mg) was added. The concentration of the product was determined by relative peak integration. The ethynyl-phosphonite was immediately used in the following reaction.

$^{31}P\{^{1}H\}$-NMR (243 MHz, DMSO-d6) δ [ppm] = 130.4.

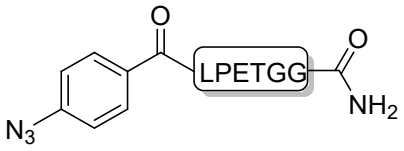
2.4 Synthesis of Ethynyl-Phosphonite 2c



Ethynyl-phosphonite **2c** was synthesized according to General Procedure I from 267 mg bis-(diisopropylamino)-chlorophosphine (1 mmol, 1 eq.) and 0.75 mL hexaethylene glycol (3 mmol, 3 eq.). The desired compound was obtained as a yellow oil. (105.52 mg, 0.15 mmol, 17%).

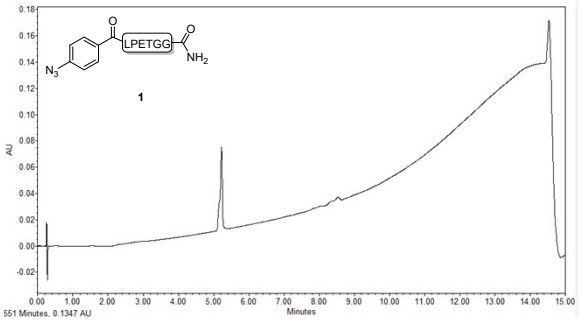
$^{31}P\{^{1}H\}$-NMR (243 MHz, DMSO-d6) δ [ppm] = 131.0.

2.5 Synthesis of Azido-Peptide 1

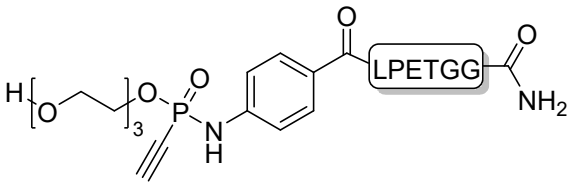


Azido-Peptide **1** was synthesized from model peptide NH_2-LPETGG on Rink amide resin (12.5 µmol, 1 eq.) and 4-azido-benzoic acid (37.5 µmol, 3 eq.). The coupling reaction was performed in 0.7 mL DMF for 1.5 hours at room temperature with coupling agents HATU (37.5 µmol, 3 eq.) and DIPEA (75 µmol, 6 eq.). The resulting azido-peptide was washed with 5 mL DMF and 5 mL CH_2Cl_2 (5x). Then, the resin was treated with 5 mL of **cocktail I** (95% TFA, 2.5% TIS, 2.5% H_2O). for 120 minutes to remove all protecting groups and to cleave the desired product off the resin. After precipitation in 45 mL cold diethylether, the product was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (3.62 mg as a TFA-salt, 5.0 µmol, 40% isolated yield).

ESI-MS: m/z calculated for $C_{45}H_{71}N_8O_{18}P$ $[M+H]^+$: 717.8, observed 717.5.



2.6 Synthesis of Ethynyl-Phosphonamidate **3a** in Solution

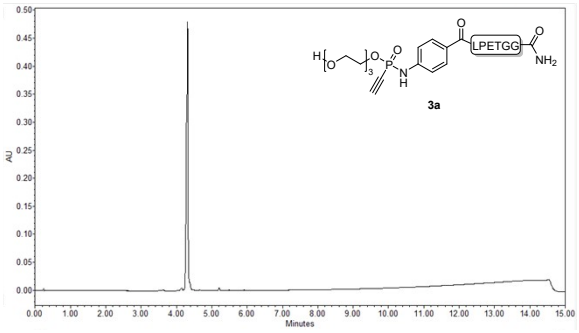


Under argon atmosphere, a 25 mL Schlenk flask was charged with 6.74 mg bis-(diisopropylamino)-chlorophosphine (25.2 µmol, 5 eq.) and cooled to 0 °C. Subsequently, 60.6 µL ethynyl-magnesium-bromide solution (0.5 M in THF, 30.3 µmol, 6 eq.) was added dropwise. The suspension was allowed to warm up to room temperature and stirred for further 40 minutes. Subsequently, 7.57 µL triethylene glycol (75.7 µmol, 15 eq.), dissolved in 168 µL 1*H*-tetrazole solution (0.45 M in

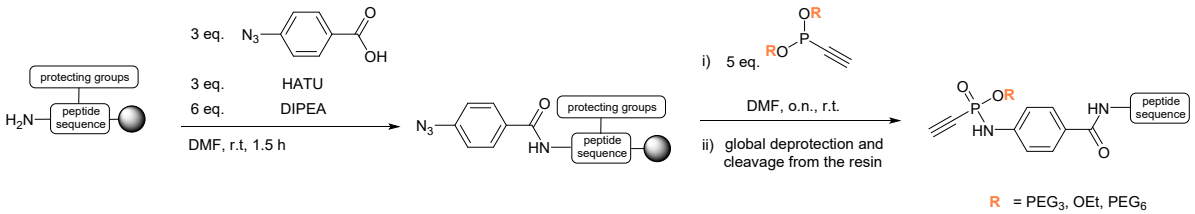
MeCN, 75.7 µmol, 15 eq.), was added and the off- white suspension was stirred for 2 hours at room temperature. 3.62 mg azido-Peptide **1** (5.05 µmol, 1 eq.) dissolved in 471 µL dry DMF were added and stirred over-night at room temperature. Solvents were removed under reduced pressure and the crude product was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and obtained as a white powder after lyophilization (1.32 mg, 1.45 µmol, 29%).

ESI-MS: m/z calculated for $C_{39}H_{59}N_8O_{15}P$ $[M+H]^+$: 911.9, observed 911.6.

HRMS (Q-Tof): calculated for $C_{39}H_{59}N_8O_{15}P$ $[M+H]^+$: 911.3910, observed 911.3912.



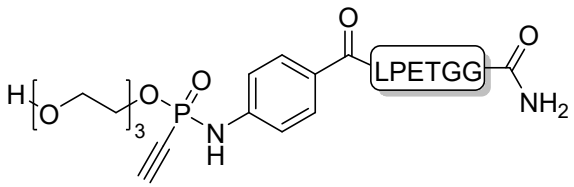
2.7 General Procedure II for the Synthesis of Ethynyl-Phosphonamidates on Resin



Ethynyl-phosphonamidates **3a-c** and **4-6** were synthesized in a 25 µmol scale on Rink amide resin (0.40 - 0.80 mmol/g, 100 – 200 mesh). In a 5 mL syringe reactor, 4-azido-benzoic acid (75 µmol, 3 eq.) was coupled to the free N-terminus of peptide **1**. The coupling reaction was performed in 0.7 mL DMF for 1.5 hours at room temperature with coupling agents HATU (75 µmol, 3 eq.) and DIPEA (150 µmol, 6 eq.). The resulting azido-peptide was washed with 5 mL DMF and 5 mL CH_2Cl_2 (5x). Next, the SPhR

was performed on resin with 5 eq. of ethynyl-phosphonite **2a**-**c** in 0.7 mL DMF over-night. Unreacted ethynyl-phosphonite was removed by washing with 5 mL DMF and 5 mL CH_2Cl_2 (5x). The resin was treated with 5 mL of **cocktail I** (95% TFA, 2.5% TIS, 2.5% H_2O). for 60 minutes to remove all protecting groups and to cleave the desired product off the resin. After precipitation in 45 mL cold diethylether, the crude ethynyl-phosphonamidate was purified by semipreparative HPLC.

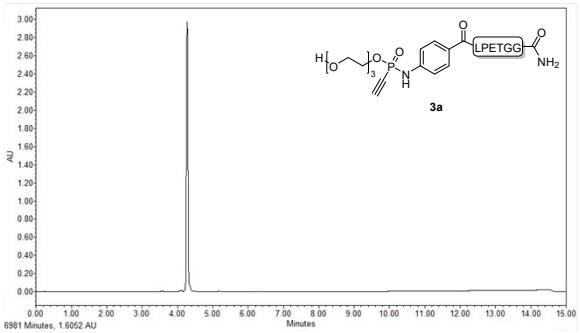
2.8 Synthesis of Ethynyl-Phosphonamidate 3a



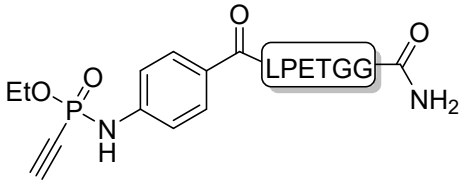
Ethynyl-phosphonamidate **3a** was synthesized according to General Procedure II from resin-bound model peptide NH_2-LPETGG (25 µmol, 1 eq.) and ethynyl-phosphonite **4a** (125 µmol, 5 eq.)**. 3a** was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (13.2 mg as a TFA-salt, 12.9 µmol, 52% isolated yield).

ESI-MS: m/z calculated for $C_{39}H_{59}N_8O_{15}P$ $[M+H]^+$: 911.9, observed 911.6.

HRMS (Q-Tof): calculated for $C_{39}H_{59}N_8O_{15}P$ $[M+H]^+$: 911.3910, observed 911.3912.



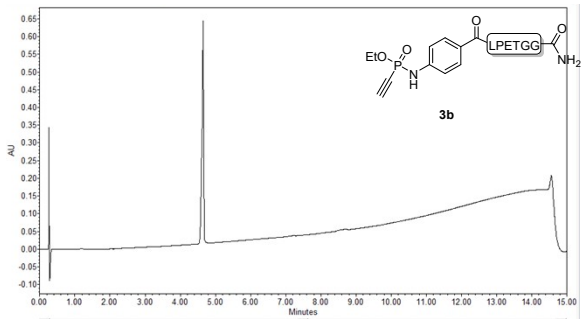
2.9 Synthesis of Ethynyl-Phosphonamidate 3b



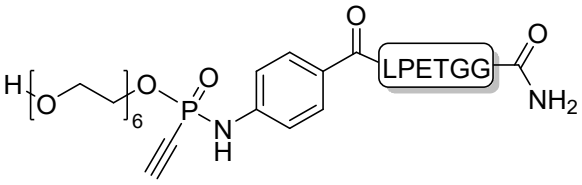
Ethynyl-phosphonamidate **3b** was synthesized according to General Procedure II from resin-bound model peptide NH_2-LPETGG (12.5 µmol, 1 eq.) and ethynyl-phosphonite **2b** (62.5 µmol, 5 eq.)**. 3b** was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (2.44 mg as a TFA-salt, 3.02 µmol, 24% isolated yield).

ESI-MS: m/z calculated for $C_{35}H_{51}N_8O_{12}P$ $[M+H]^+$: 807.8, observed 807.5.

HRMS (Q-Tof): calculated for $C_{35}H_{51}N_8O_{12}P$ $[M+H]^+$: 807.3437, observed 807.3439.



2.10 Synthesis of Ethynyl-Phosphonamidate 3c

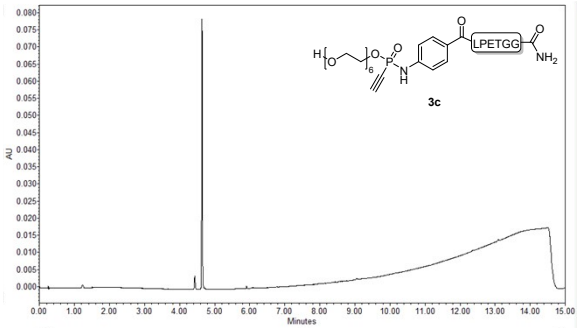


Ethynyl-phosphonamidate **3c** was synthesized according to General Procedure II from resin-bound model peptide NH_2-LPETGG (12.5 µmol, 1 eq.) and ethynyl-phosphonite **2c** (62.5 µmol, 5 eq.)**. 3c** was

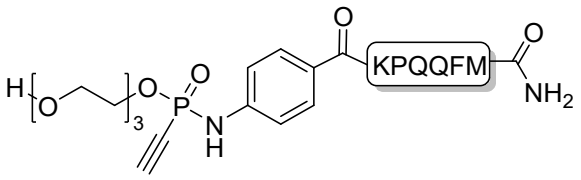
purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (3.8 mg as a TFA-salt, 3.28 µmol, 26% isolated yield).

ESI-MS: m/z calculated for $C_{45}H_{71}N_8O_{18}P$ $[M+H]^+$: 1044.0, observed 1043.8.

HRMS (Q-Tof): calculated for $C_{45}H_{71}N_8O_{18}P$ $[M+H]^+$: 1043.4697, observed 1043.4689.



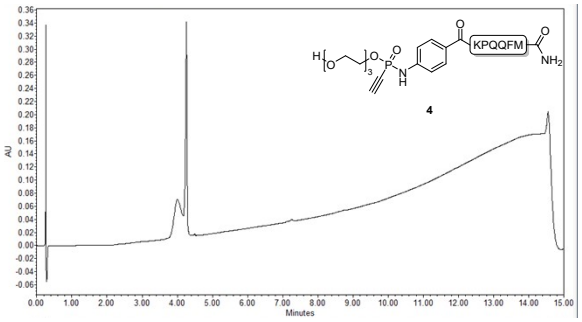
2.11 Synthesis of Ethynyl-Phosphonamidate **4**



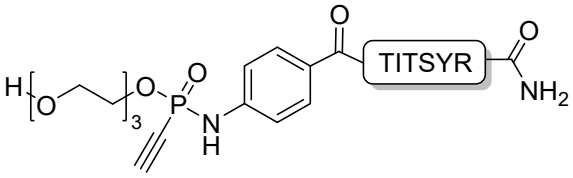
Ethynyl-phosphonamidate **4** was synthesized according to General Procedure II from model peptide NH_2-KPQQFM on Rink amide resin (12.5 µmol, 1 eq.) and ethynyl-phosphonite **2a** (62.5 µmol, 5 eq.)**.** **4** was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (4.3 mg as a TFA-salt, 3.85 µmol, 31% isolated yield).

ESI-MS: m/z calculated for $C_{50}H_{74}N_{11}O_{14}PS$ $[M+H]^+$: 1117.2, observed 1116.8.

HRMS (Q-Tof): calculated for $C_{45}H_{71}N_8O_{18}P$ $[M+H]^+$: 1116.4948, observed 1116.4985.



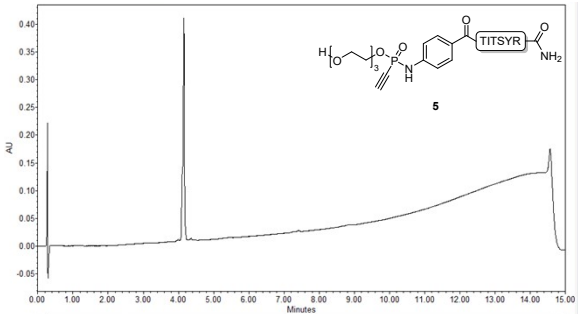
2.12 Synthesis of Ethynyl-Phosphonamidate 5



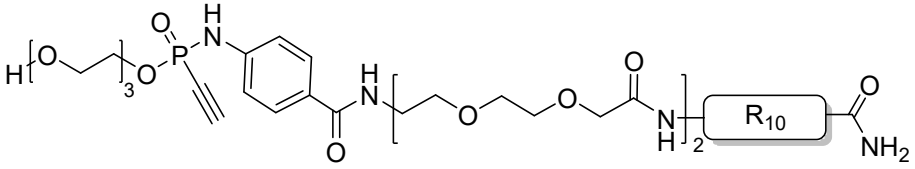
Ethynyl-phosphonamidate **5** was synthesized according to General Procedure II from resin-bound model peptide NH_2-TITSYR (12.5 µmol, 1 eq.) and ethynyl-phosphonite **2a** (62.5 µmol, 5 eq.)**. 5** was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (3.1 mg as a TFA-salt, 2.87 µmol, 23% isolated yield).

ESI-MS: m/z calculated for $C_{50}H_{74}N_{11}O_{14}PS$ $[M+H]^+$: 1079.1, observed 1078.7.

HRMS (Q-Tof): calculated for $C_{45}H_{71}N_8O_{18}P$ $[M+H]^+$: 1078.4969, observed 1078.4927.



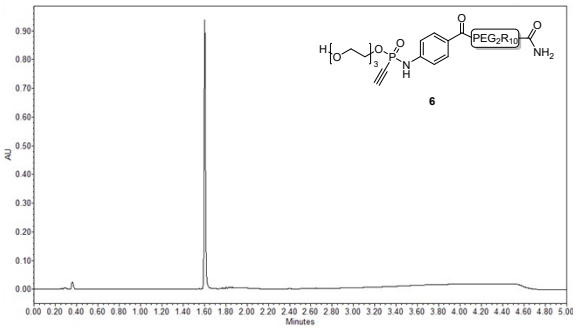
2.13 Synthesis of Ethynyl-Phosphonamidate 6



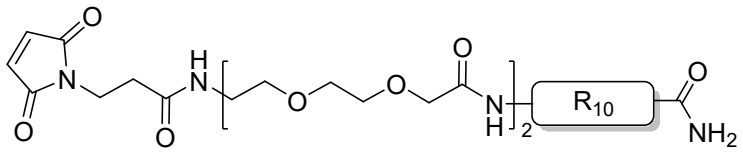
Ethynyl-phosphonamidate **6** was synthesized according to General Procedure II from resin-bound model peptide NH_2-PEG_2-R_{10} (8 µmol, 1 eq.) and ethynyl-phosphonite **2a** (40 µmol, 5 eq.)**. 6** was purified by semipreparative HPLC (5-50% B in 50 min, 50-99% in 10 min, 99% for 5 min) and gained as a white powder (3.69 mg as a TFA-salt, 1.1 µmol, 14% isolated yield).

ESI-MS: m/z calculated for $C_{87}H_{163}N_{44}O_{22}P$ ([M+3H]$^{3+}$/3) + 7 TFA: 1003.2, observed 1003.3.

HRMS (Q-Tof): calculated for $C_{87}H_{163}N_{44}O_{22}P$ ([M+3H]$^{4+}$/4): 552.8260, observed 552.8265



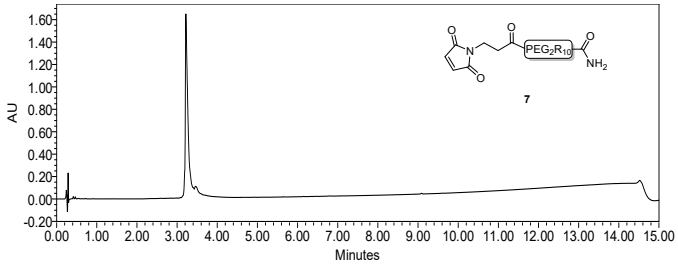
2.14 Synthesis of Maleimide-PEG_2-R10 7



Maleimide-PEG_2-R10 **6** was synthesized by reacting resin-bound model peptide NH_2-PEG_2-R_{10} (0.1 mmol, 1 eq.) and N-maleoyl-β-alanine (0.5 mmol, 5 eq.), in the presence of HATU (5 eq.) and DIEA (10 eq.). The peptide was cleaved using 95:2.5:2.5 TFA/TIPS/H_2O and was purified by semipreparative HPLC (10-50% B in 40 min, 50-95% in 2 min, 95% for 3 min) and gained as a white powder (46 mg as a TFA-salt, 22.8 µmol, 23% isolated yield).

ESI-MS: m/z calculated for $C_{79}H_{150}N_{44}O_{19}$ ([M+3H]$^{3+}$/3) + 7 TFA: 940.4, observed 940.6.

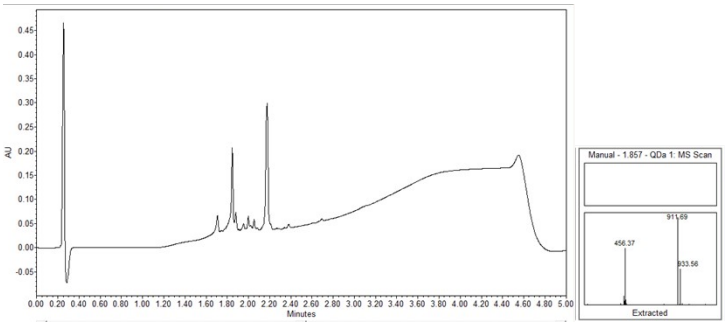
HRMS (Q-Tof): calculated for $C_{79}H_{150}N_{44}O_{19}$ ([M+3H]$^{4+}$/4): 505.8025, observed 505.8209



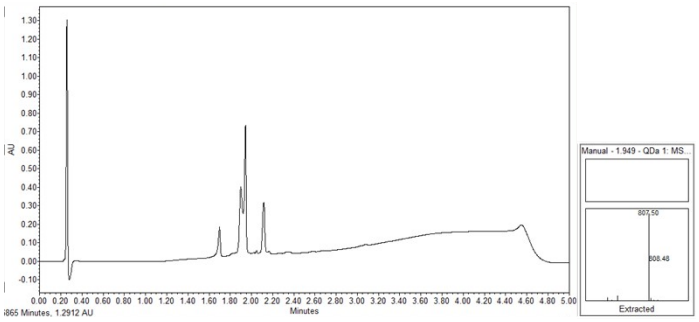
3 UPLC-ESI-MS spectra of Crude Peptides after Test Cleavage

Each of the crude UPLC-ESI-MS spectra was obtained after test cleavage in in 300 µL of cocktail I (95% TFA, 2.5% TIS, 2.5% H_2O). The test cleavages were performed at room temperature under slight agitation in the dark. Then, 400 µL of H_20/MeCN (50:50) were added and the suspension was filtered. For UPLC-ESI-MS analysis, the sample was diluted 1:3 with H_2O/MeCN (50:50).

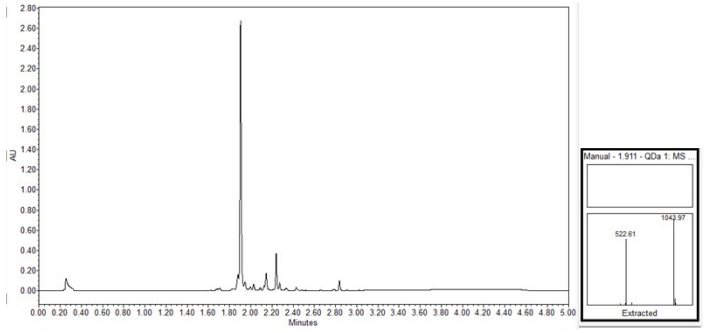
3.1 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 3a



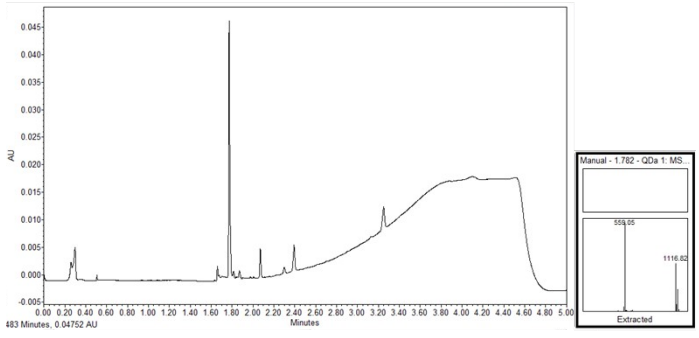
3.2 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 3b



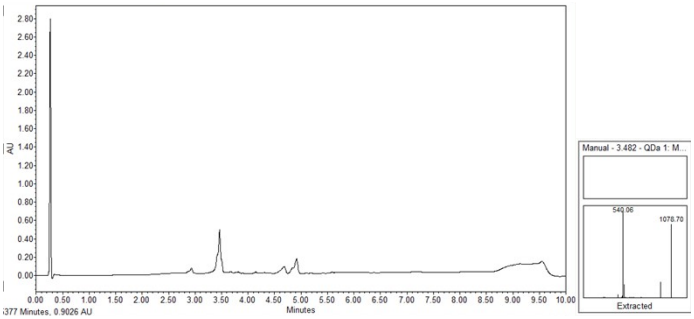
3.3 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 3c



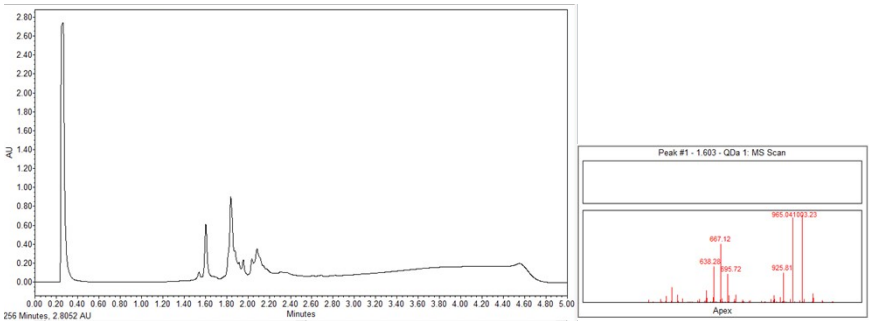
3.4 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 4



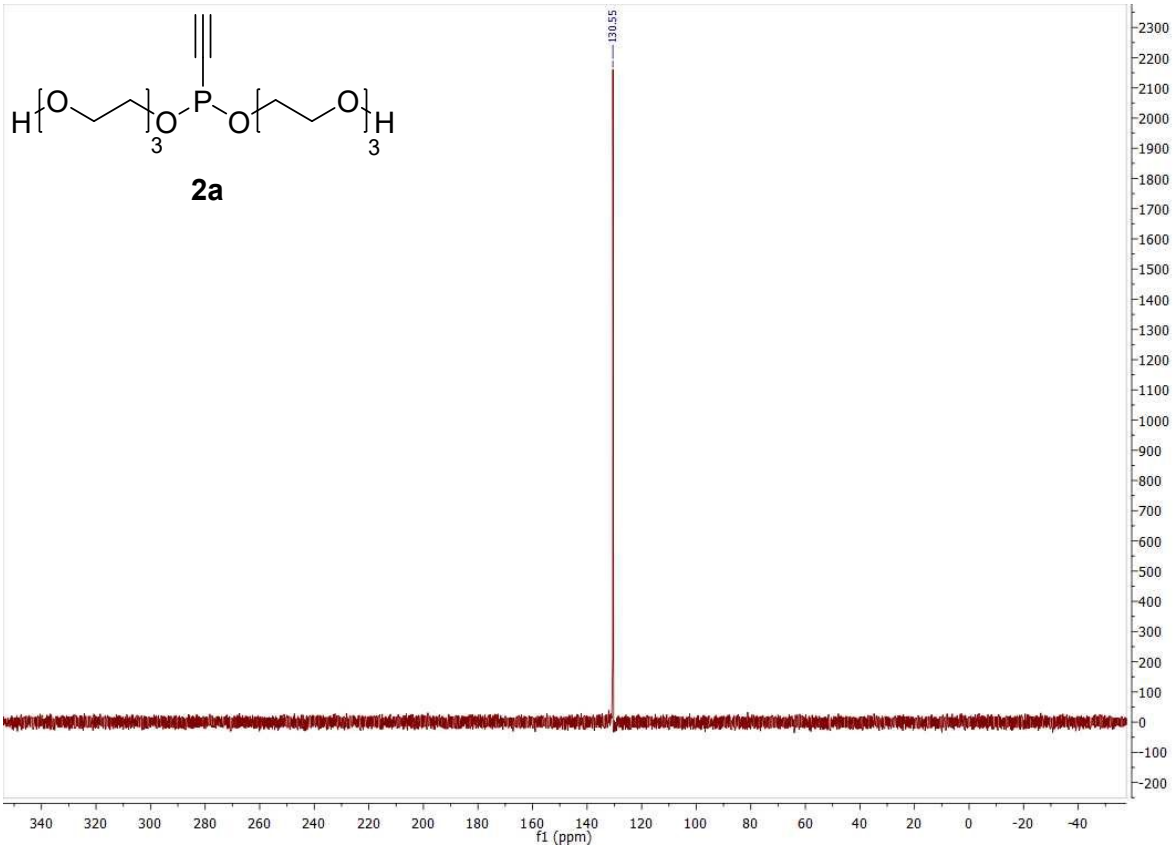
3.5 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 5



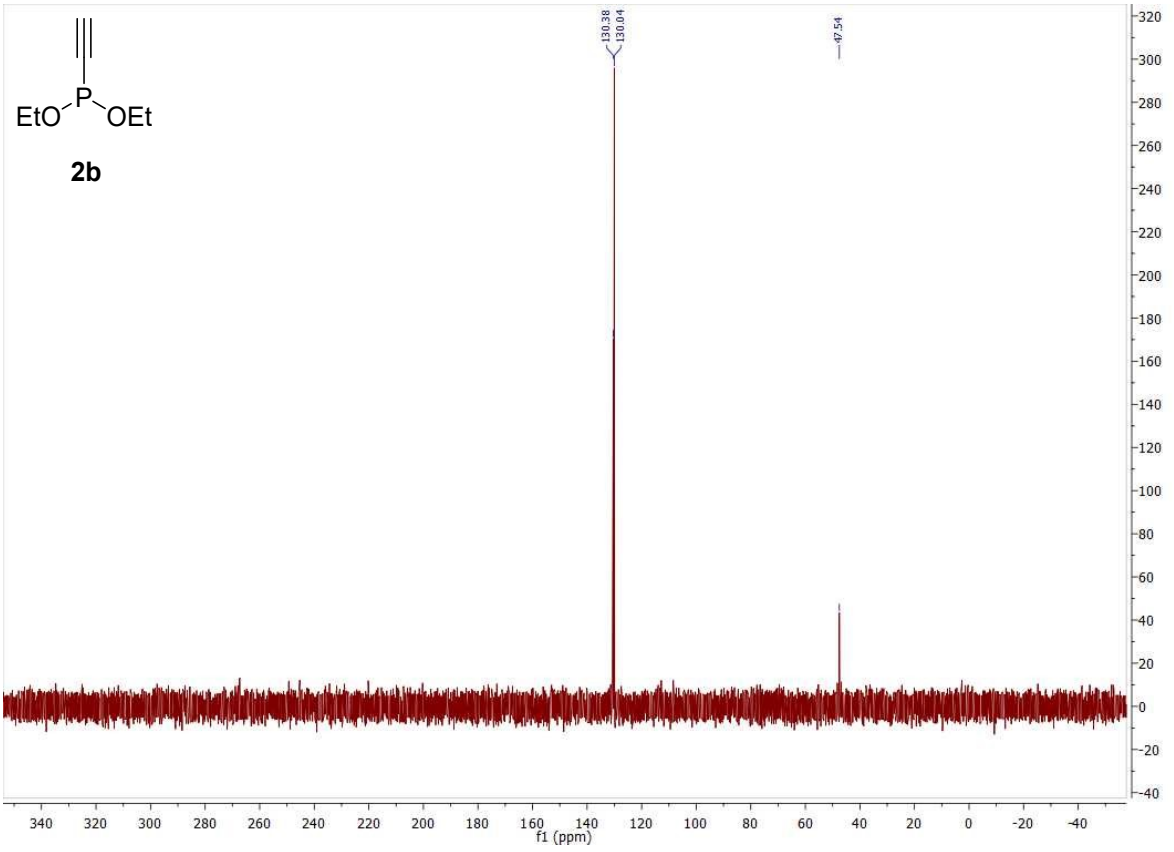
3.6 UPLC-ESI-MS spectra of Crude Ethynyl-Phosphonamidate 6



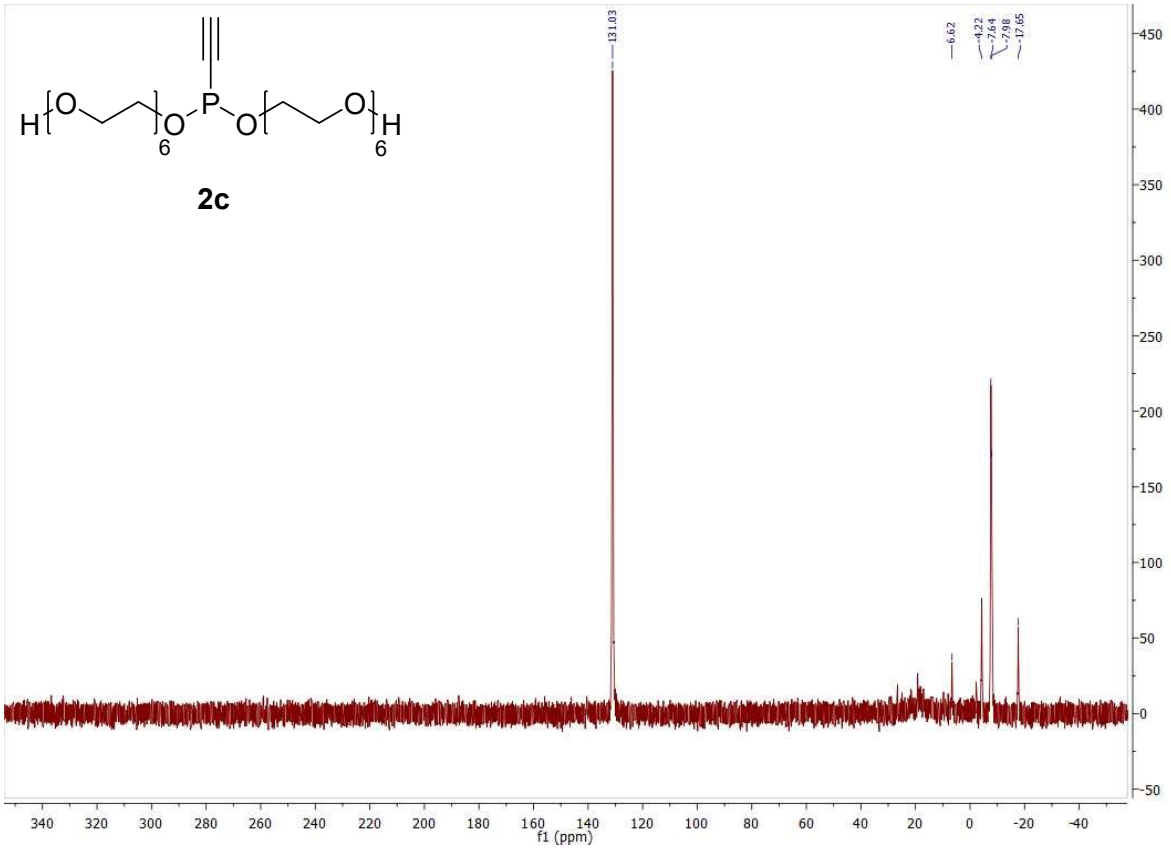
4 $^{31}P\{^{1}H\}$-NMR Spectra



2a



2b



5 References

[1] A. F. L. Schneider, M. Kithil, M. Cardoso and C. P. R. Hackenberger, *Nat. Chem.*, 2021, **12**, 530-539.

[2] M. A. Kasper, A. Stengl, P. Ochtrop, M. Gerlach, T. Stoschek, D. Schumacher, J. Helma, M. Penkert, E. Krause, H. Leonhardt and C. P. R. Hackenberger, *Angew. Chem. Int. Ed.*, 2019, **58**, 11631-11636.

[3] M. A. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso. H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.*, 2019, **58**, 11625-11630.