Traceless parallel peptide purification by a first-in-class reductively cleavable linker system featuring a safety-release

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Abbreviations

abbreviation	description
Aq.	aqueous
AUC	area under the curve
Cy	cyclohexane
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	1,3-dicyclohexylurea
DIC	*N,N*'-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DTT	dithiothreitol
EDT	ethanedithiol
eq	equivalents
GdmCl	guanidium chloride
IB	immobilisation buffer, 0.1 M sodium citrate, pH 4.5
IB(GdmCl)	0.1 M sodium citrate with 7 M guanidium chloride, pH 3.5
IS	internal standard: IS was used in this study for UPLC measurements to ensure reliable injection and reproducibility of peak integration. 0.1 mg/mL Fmoc-L-Glu(OtBu)-OH in H_2O/MeCN 17:3
MS	Mass spectrometry
NHS	*N*-hydroxysucccinimide
Oxyma	ethyl-2-cyano-2-(hydroxyimino) acetate
PS	Polystyrene
rt	Room temperature
TFA	trifluoro acetic acid
TIS	triisopropylsilane
TMSBr	trimethylsilyl bromide
t_R	retention time
UPLC	Ultra-performance liquid chromatography

S1 General information

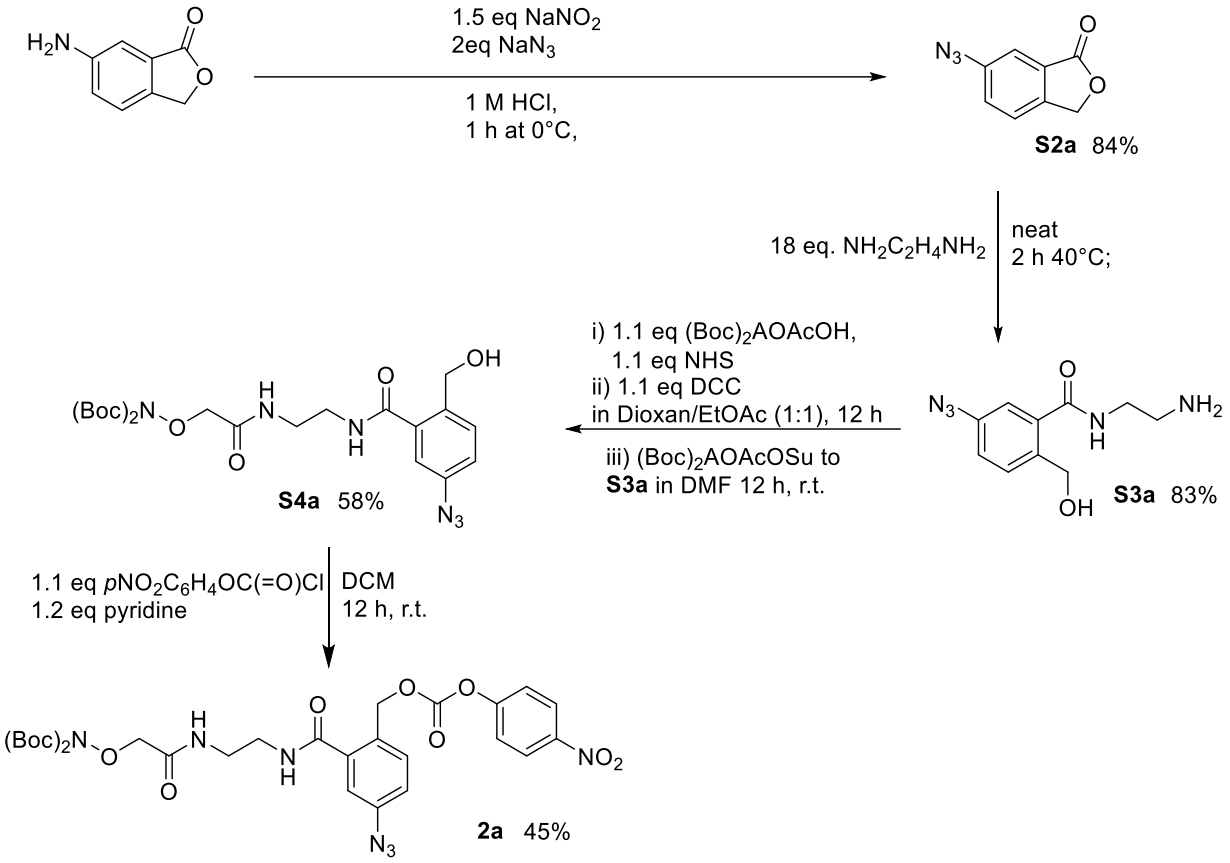
All reaction mixtures were stirred magnetically or by a KPG stirrer. The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker Advance II spectrometer (500 MHz). Chemical shifts δ are given in ppm, coupling constants J in Hz. The corresponding solvent signals were used as internal standard. Peak multiplicities are reported with the following abbreviations: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Mass spectra were measured with an analytical Acquity H-class UPLC-ESI-MS system from Waters on a C-18 column (Peptide CSH C18 Column, 130Å, 1.7 µm, 2.1 mm X 150 mm) from Waters. As mobile phase, mixtures of water (A) and acetonitrile (B) each with 0.1% TFA were used. Thin layer chromatography (TLC) was performed on silica 60 F254 plates from Merck. For detection, UV light with a wavelength of 254 nm, ninhydrin or $KMnO_4$ was used. Solvents and reagents were purchased from Carl Roth GmbH & Co. KG, Sigma Aldrich, Tokyo Chemical Industry or Suzhou Highfine Biotech Co. Ltd. Crosslinked aldehyde modified agarose beads (100 µmol aldehyde/mL settled beads) were developed for Belyntic GmbH, where they can be purchased.

S2 Synthesis of purification linkers and peptides

Base-labile linker **1a** and **1b** where synthesized and used as described by us earlier.[1]

S2.1 Synthesis of reductively cleavable linker 2a

Synthesis of reductively cleavable linker **2a** followed the reaction **Scheme S 1**.



Scheme S 1: Reaction overview for the synthesis of **2a**.

*6-azidophthalide (**S2a**)*

A clear solution of 6-aminophthalide (5.93 g, 38.76 mmol, 1 eq) in 1 M HCl (68 mL) was cooled down to 0 °C and a solution of aqueous $NaNO_2$ (4.05 g, 58.13 mmol, 1.5 eq) was added dropwise. The suspension was stirred for 10 min at 0 °C and subsequently, NaN_3 (5.09 g, 77.52 mmol, 2 eq) in 15 mL water was added dropwise. Strong formation of gases (N_2, HN_3) was observed. The foamy solution was stirred at 0°C for 1 h and filtrated in a glass-frit. The filter cake was washed with 500 mL H_2O. Subsequently, the filter cake was dissolved in CH_2Cl_2 and extracted with brine and distilled water. After drying the CH_2Cl_2 phase with $MgSO_4$, the organic solvent was removed *in vacuo* and a faint brownish solid was obtained in a yield of 84% (5.71 g, 32.6 mmol).

R_f = 0.58 (EtOAc/cyclohexane 1:1); UPLC-MS: t_R = 2.90 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 54%; ESI-MS [m/z]: calculated MH^+: 176.14; found: 176.06.

^{1}H NMR (500 MHz, DMSO-d_6) δ [ppm]: 7.71 (dt, *J* = 9.0, 0.8 Hz, 1H), 7.52 – 7.49 (m, 2H), 5.40 (s, 2H).

*N-(2-aminoethyl)-3-azido-6-(hydroxymethyl)benzamide (**S3a**)*

Lactone **S2a** (5.71 g, 32.60 mmol, 1 eq) was dissolved in ethylenediamine (39.23 mL, 586.8 mmol, 18 eq) and stirred at 40 °C until the reaction was quantitative according to UPLC-MS analysis (2 h). The ethylenediamine was removed *in vacuo.* To remove residual ethylenediamine, 100 mL H_2O was added and the remaining solid was dissolved. Subsequently, the solvents were removed under reduced pressure and the yield of **S3a** was determined to be 8.94 g (38.00 mmol, 117% - water still inside). 8.05 g (90%) of the product was hence washed with cold Brine solution to remove residual ethylenediamine, followed by a quick flush with cyclohexane. The compound was dissolved in methanol, transferred to a 250 mL flask and the solvent was removed *in vacuo*. The yield after the washing step was 83% (5.71 g, 24.27 mmol, 10% were taken off for a test). R_f = 0.1 (DCM/MeOH 8:2); UPLC-MS: t_R = 1.80 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 89%; ESI-MS [m/z]: calculated MH^+: 236.25; found: 236.17.

*N-(2-(2-bis-(tert-butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-6-(hydroxymethyl)benzamide (**S4a**)*

A solution of *N*,*N*'-bis-Boc-amino-oxyacetic acid (18.75 g, 64.40 mmol) in 220 mL dioxane/EtOAc (1/1) was cooled to 0 °C, and *N*-hydroxysuccinimide (7.41 g, 64.40 mmol) and dicyclohexylcarbodiimide (13.29 g, 64.40 mmol) were added. The mixture was stirred at rt overnight. The suspension was filtrated over celite (20 g) and washed with ethyl acetate. The solvents were removed *in vacuo* and the *N*,*N*'-bis-Boc-amino-oxyacetic succinimide was obtained as white/yellowish solid. The yield of the intermediate was 96% (23.96 g, 61.73 mmol). **S3a** (5.71 g, 24.27 mmol, 1.05 eq) was dissolved in 120 mL DMF (slow dissolution) and the succinimide-ester (8.97 g, 23.11 mmol, 1 eq) was added as a solid. The reaction mixture was stirred at rt for approx. 12 h and completion was checked by UPLC-MS. DMF was removed by co-evaporation with toluene at 60 °C and under high-vacuum (72 h). The compound was then dissolved in ethyl acetate and extracted 3x with 5% $NaHCO_3$ (each 200 mL), 3x 2% citric acid solution at pH 4.5 (3x 200 mL) and water (3x 200 mL). The organic layer was dried over $MgSO_4$ and the organic solvent was removed *in vacuo*. **S4a** was obtained as an orange amorphous solid with a yield of 58% (7.18 g, 14.12 mmol).

R_f = 0.45 (DCM/MeOH 19:1); UPLC-MS: t_R = 3.21 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 54%; ESI-MS [m/z]: calculated MH^+: 531.22, 229.96; found: 531.08.

*2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-benzyl-(4-nitrophenyl) carbonate (**2a**)*

The benzyl alcohol **S4a** (7.18 g, 14.12 mmol, 1.0 eq) was provided in CH_2Cl_2 (30 mL) (dry if possible) and (dry) pyridine (1.37 mL, 16.94 mmol, 1.2 eq) was added under ice-cooling. 4-nitrophenylchloroformiate (3.13 g, 15.53 mmol, 1.1 eq) dissolved in 20 mL CH_2Cl_2 was added slowly and the reaction mixture was stirred overnight. LC-MS and TLC analysis indicated a complete reaction after approx. 12 h. Dichloromethane was removed *in vacuo* and the crude mixture was purified by flash column chromatography (approx. 500 g silica, 2 L EtOAc and 2 L cyclohexane) with a gradient of 1 L of Cy/EtOAc 2:1, followed by 3 L of a Cy/EtOAc 1:1. After removal of the organic solvents, the product was obtained as an off-white solid with a yield of 4.26 g (6.32 mmol, 45%).

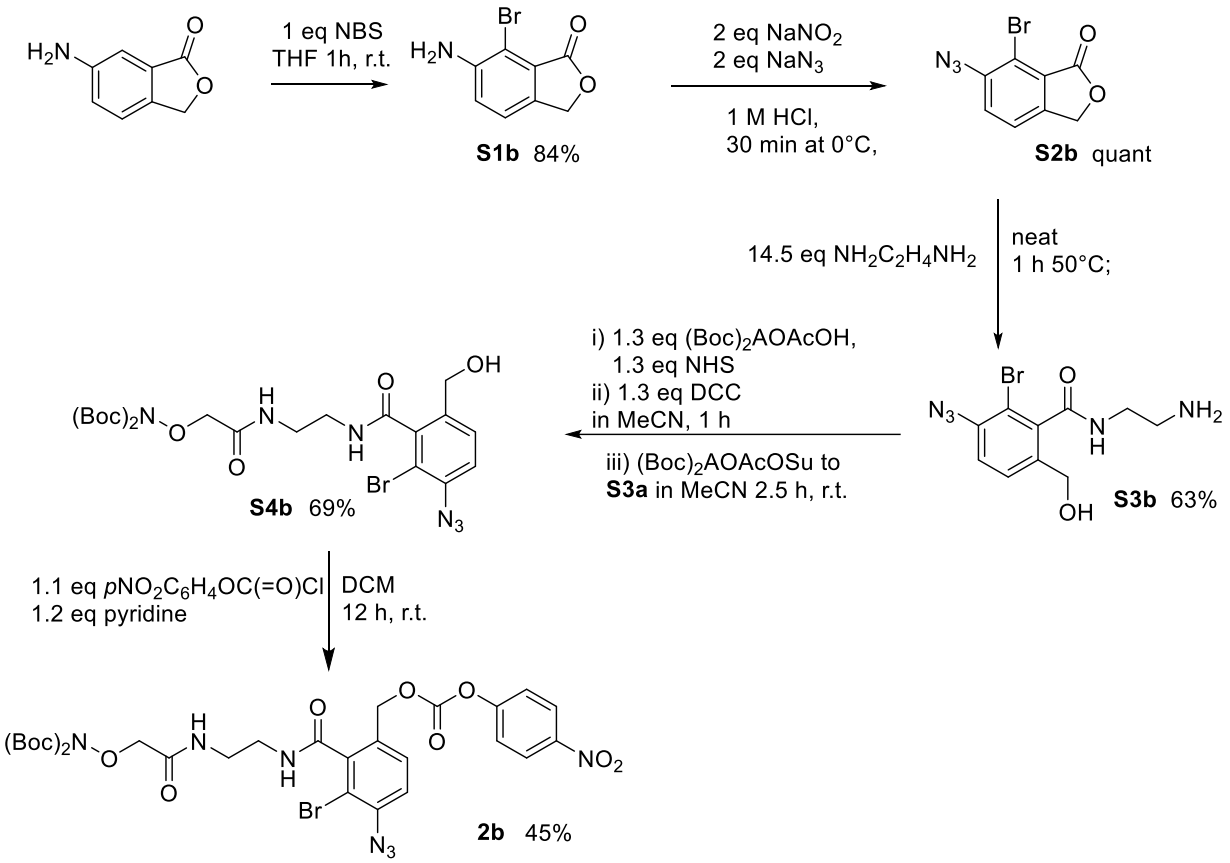
R_f = 0.51 (Cyclohexane/EtOAc 1:1); UPLC-MS: t_R = 3.55 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 92%; ESI-MS [m/z]: calculated MNa^+: 696.22; found: 696.21; elemental analysis: (calculated C, 51.71; H, 5.24; N, 14.56; O, 28.50; found: C, 51.73; H, 5.17; N, 14.52).

^{1}H NMR (500 MHz, DMSO-d_6) δ [ppm]: 8.63 (t, *J* = 5.2 Hz, 1H), 8.32 (d, *J* = 9.2 Hz, 2H), 8.04 (s, *J* = 5.5 Hz, 1H), 7.56 (d, *J* = 9.2 Hz, 2H), 7.58 (m, 1H), 7.27 (m, 2H), 5.45 (s, 2H), 4.36 (s, 2H), 3.32 (m, 4H), 1.45 (s, 18H).

^{13}C NMR (126 MHz, DMSO-d_6) δ [ppm]: 167.11, 166.54, 155.30, 151.80, 149.31, 145.17, 139.58, 137.18, 130.90, 129.82, 125.44, 122.56, 120.52, 118.56, 83.87, 74.95, 67.72, 38.91, 37.85, 27.53.

S2.2 Synthesis of reductively cleavable linker 2b

Synthesis of reductively cleavable linker **2b** followed reaction Scheme S 2.



Scheme S 2: Reaction overview for the synthesis of **2b**.

*6-Amino-7-bromophthalide (**S1b**)*

To a cooled solution (0 °C) of 6-aminophthalide (5.13 g, 34.05 mmol) in THF (80 mL) was added *N*-bromosuccinimide (6.12 g, 34.05 mmol, 1 eq). The cooling bath was removed, and the solution was stirred for 1 h. The solvent was removed under reduced pressure. The yellow residue was taken up in ethyl acetate (400 ml) and washed three times with water (200 mL each). The organic phase was dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give 6-amino-7-bromophthalide as a brown solid (6.53 g, 28.63 mmol, 84%). R_f = 0.2 (cyclohexane/ethyl acetate 2:1); UPLC-MS: t_R = 1.45 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 83.1%; ESI-MS [m/z]: calculated MH^+: 227.97, 229.96; found: 228.01, 230.01.

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm]: 7.31 (d, *J* = 8.2 Hz, 1H), 7.16 (d, *J* = 8.2 Hz, 1H), 5.74 (s, 2H), 5.16 (s, 2H).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm]: 168.8, 147.2, 137.3, 123.2, 122.0, 121.6, 101.7, 67.7.

*6-azido-7-bromophthalide (**S2b**)*

6-Amino-5-bromophthalide (5.54 g, 24.17 mmol) was added to 0 °C cold hydrochloric acid (1 M, 100 mL). To the cooled suspension, conc. sulfuric acid was added dropwise whilst stirring until the solid was completely dissolved (25 mL). The solution was further cooled until it reached 0 °C again. A solution of sodium nitrite (3.34 g, 48.34 mmol, 2 eq.) in water (17 mL) was added slowly (formation of nitrous gases if solution too warm). After stirring for 10 minutes, a solution of sodium azide (3.14 g, 48.34 mmol, 2 eq) in water (20 mL) was slowly added dropwise (caution: formation of hydrazoic acid). After 30 min, the suspension was extracted with ethyl acetate (200 mL). The aqueous phase was filtered, and the filter cake was washed three times with water (100 mL each) and once with cyclohexane (150 mL). 6-azido-7-bromophthalide (6.58 g, 24.17 mmol, quant.) was obtained as a yellow solid. R_f = 0.3 (cyclohexane/ethyl acetate 2:1); UPLC-MS: t_R = 2.24 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 50.3%.

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm]: 7.76 (d, *J* = 8.1 Hz, 1H), 7.70 (d, *J* = 8.2 Hz, 1H), 5.32 (s, 2H).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm]: 167.68, 146.04, 139.52, 126.02, 124.72, 123.17, 110.06, 67.99, 39.52.

*N-(2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-2-bromo-6-(hydroxymethyl) benzamide (**S3b**)*

6-Azido-7-bromophthalide (5.54 g, 24.17 mmol) was taken up in acetonitrile (150 mL) and the suspension was heated to 50 °C whilst stirring. Ethylenediamine (23.4 mL, 350.47 mmol, 14.5 eq.) was added and the solid completely dissolved within 10 min. After 1 h stirring at 50 °C, the solvent and excess ethylenediamine were removed under reduced pressure to give a red oil (8.49 g). Saturated brine (80 mL) was added, the resulting suspension was sonicated for 30 min, stirred at 40 °C for 30 min, and filtered. The filter cake was washed once with saturated brine (50 mL) and once with cyclohexane (100 mL). After drying the filter cake, the title compound was obtained as a yellow solid (3.84 g, 12.2 mmol, 50.6%). Product **3**b was also obtained from the filtrate by extraction with ethyl acetate (six times with 150 mL each time) (4.79 g, 15.22 mmol, 63.1%). R_f = 0.1 (DCM/MeOH 8:2); UPLC-MS: t_R = 1.03 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (210 nm) = 83.5%; ESI-MS [m/z]: calculated MNa^+: 336.01, 338.01; found: 335.95, 337.96.

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm]: 8.45 (t, *J* = 5.4 Hz 1H), 7.55 (d, *J* = 8.3 Hz, 1H), 7.43 (d, *J* = 8.3 Hz, 1H), 4.43 (s, 2H), 3.22 (m, 2H), 3.22 (dd, *J* = 6.1 Hz, *J* = 12.0 Hz, 4H), 2.67 (t, *J* = 6.3 Hz, 2H).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm]: 165.90, 139.48, 137.50, 136.61, 127.35, 119.57, 110.28, 59.85, 42.45, 41.08, 39.52.

*N-(2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-2-bromo-6-(hydroxymethyl) benzamide (**S4b**)*

Dicyclohexylcarbodiimide (DCC, 3.30 g, 15.86 mmol, 1.3 eq.) was added to a stirred solution of *bis-(tert-butoxycarbonyl)*-(aminooxy)acetic acid ($(Boc)_2$AOAc-OH, 4.71 g, 15.86 mmol, 1.3 eq.) and NHS (1.84 g, 15.86 mmol, 1.3 eq.) in acetonitrile (40 mL). After stirring for 1 h, the solution was separated from the resulting white precipitate by filtration and the filter cake was washed with acetonitrile (40 mL). The filtrate was diluted to 120 mL with acetonitrile. **S3b** (3.79 g, 12.06 mmol, 1 eq) was taken up in acetonitrile (30 mL) and the suspension was sonicated for 50 min. The filtrate with $(Boc)_2$AOAc-NHS was added to this suspension and the reaction mixture was stirred for 2.5 h. After removal of the solvent under reduced pressure, ethyl acetate (150 ml) was added to the resulting orange oil (10.47 g) and the suspension was sonicated for 10 min and then stirred at 50 °C for 10 min. The suspension was washed 3x with 80 mL 5 w% $NaHCO_3$ solution (pH 8), once with 80 mL 2% citric acid solution (pH 4.5) and twice with 80 mL brine. The organic phase was separated and dried over $MgSO_4$. The solvent was removed under reduced pressure, giving a yellow foam as crude product (6.84 g). After drying the crude product in high vacuum, the product was obtained as a yellow solid (6.43 g, 8.31 mmol, 68.91% yield). R_f = 0.15 (DCM/MeOH 95:5); UPLC-MS: t_R = 2.60 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (21 nm) = 79.1%; ESI-MS [m/z]: calculated MNa^+: 609.13, 611.13; found: 609.03, 611.06.

^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 8.50 (t, *J* = 5.2 Hz, 1H), 7.95 (t, *J* = 5.5 Hz, 1H), 7.57 (d, *J* = 8.4 Hz, 1H), 7.45 (d, *J* = 8.3 Hz, 1H), 4.44 (s, 2H), 4.36 (s, 2H), 3.31 (m, 4H), 1.47 (s, 18H).

^{13}C NMR (75 MHz, DMSO- d_6): δ [ppm]: 166.42, 165.98, 149.29, 138.91, 137.51, 136.58, 127.10, 119.72, 110.21, 83.89, 83.84, 74.87, 59.75, 39.52, 38.43, 37.69, 27.56.

*2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-3-bromobenzyl (4-nitrophenyl) carbonate (**2b**)*

N-(2-(2-bis-(*tert*-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-2-bromo-6-(hydroxymethyl) benzamide (6.39 g, 8.26 mmol) was dissolved in DCM (20 mL) and cooled to 0 °C. Whilst stirring, anhydrous pyridine (1.00 mL, 12.48 mmol, 1.5 eq.) was added, followed by a solution of *p*-nitrophenyl chloroformate (2.52 g, 12.48 mmol, 1.5 eq.) in DCM (20 mL). The reaction mixture was warmed to rt and stirred for 1 h. The solvent was removed and the resulting orange oil (9.99 g) was dissolved in 150 mL ethyl acetate. The suspension was filtered, and the solvent was removed from the filtrate under reduced pressure to give a yellow foamy solid as crude product

(8.85 g). After purification by column chromatography (silica gel, cyclohexane/ethyl acetate 2:1 to 1:1), the product (3.82 g) was taken up in 100 mL diethyl ether, treated with ultrasound for 10 min, stirred for 30 min at 40 °C and stored overnight at -20 °C. The product was filtered and washed with 100 mL -20 °C cold diethyl ether. After drying in high vacuum, the product was obtained as a pale-yellow solid (2.47 g, 3.29 mmol, 39.5%).

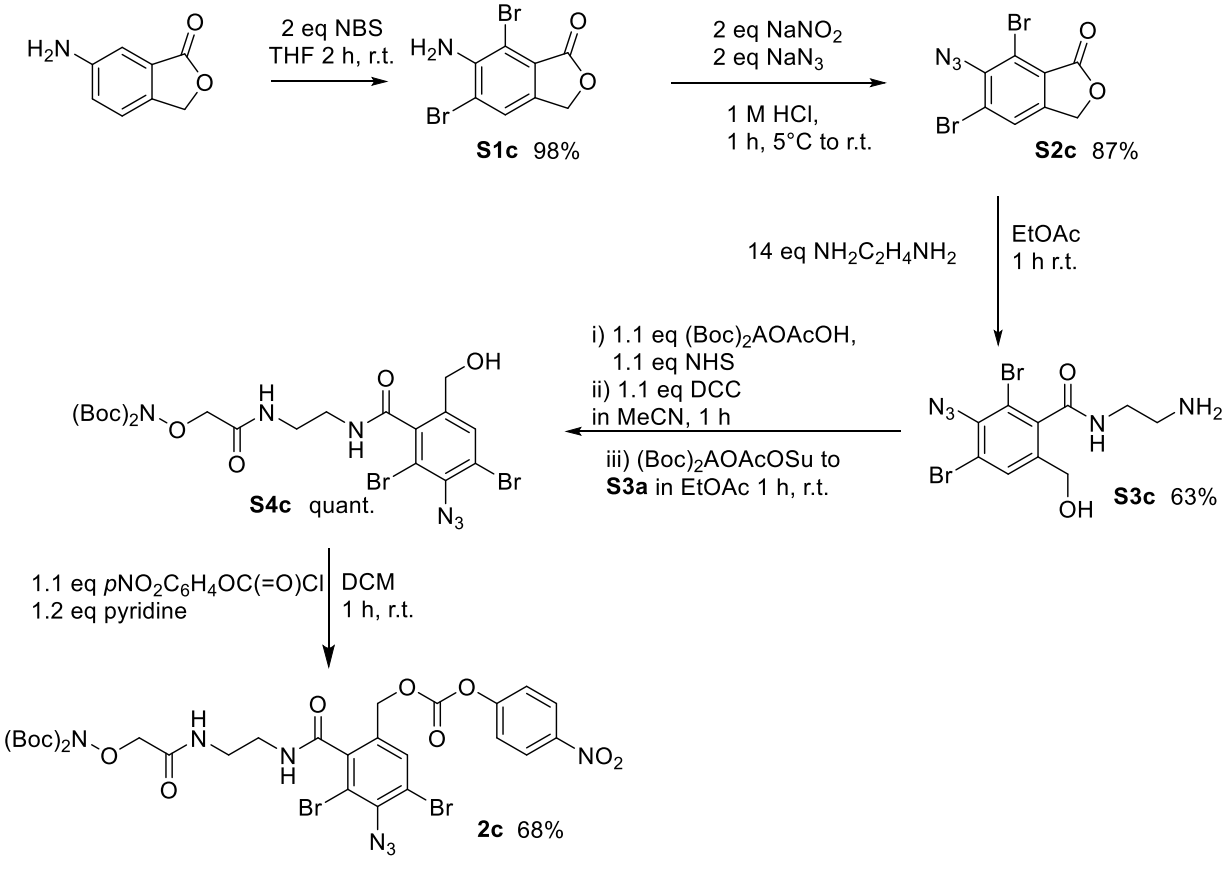
R_f = 0.25 (Ethyl acetate/cyclohexane 2:1), UPLC-MS: t_R = 3.18 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min), UPLC-purity (278 nm) = 88.4%; ESI-MS [m/z]: calculated MNa^+: 774.13, 776.13; found: 773.91, 775.88.

^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 8.71 (t, *J* = 5.1 Hz, 1H), 8.32(d, *J* = 9.2 Hz, 2H), 7.95 (t, *J* = 5.2 Hz, 1H), 7.62 (d, *J* = 8.3 Hz, 1H), 7.57 (d, *J* = 9.2 Hz, 2H), 7.52 (d, *J* = 8.3 Hz, 1H), 5.25 (s, 2H), 4.36 (s, 2H), 3.33 (m, 4H), 1.46 (s, 18H).

^{13}C NMR (75 MHz, DMSO- d_6) δ [ppm] = 166.44, 165.60, 155.24, 151.70, 149.28, 145.20, 141.08, 139.14, 130.05, 129.28, 126.18, 125.41, 122.56, 120.08, 115.78, 110.79, 83.88, 74.87, 67.38, 39.52, 38.55, 37.63, 27.53.

S2.3 Synthesis of reductively cleavable linker 2c

Synthesis of reductively cleavable linker 2c followed the reaction Scheme S 3.



Scheme S 3: Reaction overview for the synthesis of **2c**.

*5,7-Dibromo-6-aminophthalide (**S1c**)*

550 mL THF and 30 mL MeCN was added was added to 6-aminophthalide (20.00 g, 132.75 mmol) in a 1 L round bottom flask at 0°C. Whilst stirring, *N*-bromosuccinimide (47.73 g, 265.50 mmol, 2 eq) was added slowly to the solution giving a brownish color. . The ice-bath was removed, and the solution became yellow over time. After 2 h stirring at rt, UPLC-MS and TLC analysis indicated complete conversion to the dibromide. The solvent was removed under reduced and the residual solid was dissolved in 600 mL ethyl acetate. The organic layer was washed 3x with water. and dried over $MgSO_4$. After solvent evaporation, 39.86 g (129.86 mmol, 98%) of the desired product was obtained as a pale-yellow solid. R_f = 0.6 (cyclohexane/Ethyl acetate 2:1); UPLC-MS: t_R = 2.36 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (254 nm) = 87.0%; ESI-MS [m/z]: calculated MH^+: 307.95; found: 307.76.

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm]: 7.78 (s, 1H), 5.72 (s, 2H), 5.17 (s, 2H); ^{13}C NMR (101 MHz, DMSO) δ [ppm]: 168.13, 143.96, 137.47, 125.76, 123.05, 114.80, 102.80, 67.26, 39.52.

*5,7-Dibromo-6-azidophthalide (**S2c**)*

5,7-Dibromo-6-aminophthalide (38.50 g, 124.18 mmol) was dissolved in 200 mL concentrated H_2SO_4 in a 2 L flask. The brown solution was cooled with a big ice bucket and 235 mL 1 M HCl was added slowly, producing a precipitate. $NaNO_2$ (17.31 g, 248.35 mmol, 2 eq) was dissolved in 32 mL water and was added slowly to the suspension at 5 °C. The precipitate dissolved. The solution was stirred for another 15 min at 0 °C and NaN_3 (16.31 g, 248.35 mmol, 2 eq) dissolved in 75 mL water was added dropwise. Strong formation of gases (N_2, HN_3) was observed. The foamy solution was stirred for 1 h and 500 mL water was added under cooling with ice. After foam-building had ceased and the solution had reached rt, the suspension was filtered through a Büchner funnel. 2 L of water was used to transfer the solid into the funnel and to wash it simultaneously. After drying the wet product in a crystallizing dish under reduced pressure, the product was yielded as a slightly brownish solid (36.01 g, 108.16 mmol, 87.1%). R_f = 0.45 (cyclohexanes/ethyl acetate 2:1); UPLC-MS: t_R = 2.89 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (254 nm) = 95.3%.

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm]: 8.05 (s, 1H), 5.31 (d, 2H).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm]: 167.16, 147.81, 147.49, 127.13, 124.94, 124.32, 116.33, 67.56, 39.52.

*N-(2-aminoethyl)-3-azido-2,4-dibromo-6-(hydroxymethyl)benzamide (**S3c**)*

5,7-Dibromo-6-azidophtalide (18.00 g, 53.52 mmol) was dissolved in ethyl acetate (490 mL). Insoluble impurities were filtered off and ethylenediamine (52.73 mL, 749.32 mmol, 14 eq) was added at 0 °C. The reaction mixture was stirred at room temperature for 1 h, after which UPLC-MS analysis indicated quantitative conversion. The reaction mixture was transferred into a separation funnel and 100 mL brine was added. After separation of the aqueous phase, the organic layer was dried over $MgSO_4$ and the desired product was obtained after evaporation of the organic solvent as an orange solid (20.50 g, 52.16 mmol, 97.4%). R_f = 0.25 (DCM/MeOH 8:2); UPLC-MS: t_R = 1.80 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (254 nm) = 84.2%; ESI-MS [m/z]: calculated MH^+: 393.93; found: 393.87.

*N-(2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-2,4-dibromo-6-(hydroxymethyl)benzamide (**S4c**)*

$(Boc)_2NOAcOH$ (17.30 g, 58.18 mmol, 1.1 eq.) and NHS (6.76 g, 58.18 mmol, 1.1 eq.) was dissolved in 350 mL acetonitril. DCC (12.13 g, 58.18 mmol, 1.1 eq.) was added and, after dissolution of DCC, a white precipitate had formed. The reaction mixture was stirred at room temperature for 1 h . According to UPLC-MS, the $(Boc)_2NOAc$-NHS ester was formed quantitatively. The mixture was filtered into a 1 L flask to remove DCU. *N*-(2-aminoethyl)-3-azido-2,4-dibromo-6-(hydroxymethyl)benzamide (20.5 g, 52.90 mmol) was dissolved in 530 mL ethyl acetate and added to the $(Boc)_2NOAc$-NHS solution . The mixture was stirred at room temperature for 1 h, after which the completion of the reaction was confirmed by TLC and UPLC-MS analysis. Additionally, formed precipitate was filtered off and the organic phase was washed 2x with 5% $NaHCO_3$ (each 200 mL), 1x with brine and 2x with 1:1 2% citric acid solution (pH 4.5)/brine (each 150 mL). The organic phase was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The title compound was obtained as a pale yellow oil (37.70 g, 56.58 mmol, quantitative). R_f = 0.4 (DCM/MeOH 95:5); UPLC-MS: t_R = 2.97 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); UPLC-purity (254 nm) = 54.2%; ESI-MS [m/z]: calculated MNa^+: 689.04; found: 688.95.

*2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-3,5-dibromobenzyl(4-nitrophenyl) carbonate (**2c**)*

N-(2-(2-bis-(tert-butoxycarbonyl)-(aminooxy)acetamido)ethyl)-3-azido-2,4-dibromo-6-(hydroxymethyl)benzamide (37.70 g, 52.90 mmol, 1 eq) was provided in CH_2Cl_2 (170 mL) and dry pyridine (4.72 mL, 58.50 mmol, 1.1 eq) was added. 4-nitrophenylchloroformiate (12.03 g, 58.50 mmol, 1.1 eq) was added slowly as solid at room temperature keeping the temperature constant by usage of a water bath. LC-MS and TLC analysis indicated complete reaction after 1 h. Dichloromethane was removed *in vacuo* yielding 52 g crude brown oil. The

residue was dissolved in 500 mL ethyl acetate and washed 2x with 1:1 2% citric acid solution (pH 4.5)/brine (each 250 mL) and 1x with 150 mL brine. The organic phase was dried over $MgSO_4$. This suspension was filtered over a 50 g silica plug in a glass frit, whereas the orange and reddish impurities remained on the silica. The organic solvent was removed under reduced pressure till a highly viscous, slightly amber oil remained. 70 mL of Et_2O was added to this oil and the biphasic emulsion was mixed on a rotary evaporator at 45 °C for 10 min, until one homogeneous phase was formed. A small sand grain was added to the flask to initiate crystallization and the flask was put into a refrigerator overnight (16 h). Another 200 mL chilled diethyl ether was added to the formed precipitate and the mixture was gently stirred in an ice-bath. The precipitate was transferred into a Büchner funnel and washed with additional 200 mL cold Et_2O. After drying under high vacuum the desired compound was obtained as white solid (29.95 g, 36.02 mmol, 68.1%). R_f = 0.6 (ethyl acetate/cyclohexane 2:1); UPLC-MS: t_R = 2.86 min (gradient 30-95% B in 3 min, 95% B from 3-4 min); UPLC-purity (278 nm) = 93.5%; ESI-MS [m/z]: calculated MNa^+: 854.04; found: 853.87.

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm]: 8.73 (t, *J* = 5.0 Hz, 1H), 8.33 (d, *J* = 9.1 Hz, 2H), 7.95 (d, *J* = 4.8 Hz, 1H), 7.93 (s, 1H), 7.57 (d, *J* = 9.1 Hz, 2H), 5.24 (s, 2H), 4.36 (s, 2H), 3.34 (m, 4H), 1.46 (s, 18H).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm]: 166.47, 164.92, 155.19, 151.57, 149.29, 145.22, 140.22, 136.46, 133.48, 131.95, 125.40, 122.55, 117.94, 116.58, 83.89, 74.86, 66.60, 39.52, 38.56, 37.60, 27.52.

S2.4 Peptide synthesis

Table S 1. Number, sequence, origin and molecular weight (Mw) of all peptides mentioned in the main text.

Number	Peptide	Origin	M_W (Da)
P1	H-TRYQAKPVNRSTPISTGKEG-OH	research peptide	2188.17
P2	H-RTGKLAPSFNGKSSQTREIL-OH	research peptide	2188.21
P3	H-DSAPNPVLDIDGEKLRTGTN-OH	Miraculin (1-20)	2110.07
P4	H-ARTKQTARKSTGGKA-OH	Histone H3 (1-15)	1558.90
P5	H-AKADEVSLHKWYG-NH_2	research peptide	1501.77
P6	H-GWVKPIIIGHHAYGDQYRAT-NH_2	IDH1-001[2]	2280.19
P7	H-TLYEQEIEV-NH_2	GLT25D2-001[2]	1121.56
P8	H-HGSRKNITDMVEGAKKANG-NH_2	02-M08[2]	2011.04
P9	H-SLLNQPKAV-NH_2	CLSP-001[2]	967.58
P10	H-EDPYLFELPVLKYLDMGTT-NH_2	04-M01[2]	2242.12
P11	H-ALAVLSNYDA-NH_2	EGFR-007[2]	1034.54
P12	H-TMEDKIYDQQVTKQCLCF-NH_2	06-M01[2]	2191.01
P13	H-YSYPETPLYMQTASTSYYE-NH_2	07-M01[2]	2291.99
P14	H-KVGYTERQRWDFLSEASIM-NH_2	07-M04[2]	2314.15
P15	H-RLRMREHMMKNVDTNQD-NH_2	08-M01[2]	2172.05
P16	H-VYEKNGYIYF-NH_2	MMP13-001[2]	1293.64
P17	H-ALAVLCNYDA-NH_2	EGFR-007-S6C[2]	1050.52
P18	H-ALVPPSKRKMWVVSPAEKA-NH_2	13-M01[2]	2092.20
P19	H-ISTPTPTIVHPGSLPLHLG-NH_2	13-M06[2]	1935.09
P20	H-IVQENNTPGTYLLSVSARD-NH_2	14-M03[2]	2075.06
P21	H-RFHMKVSVYLLAPLREALS-NH_2	14-M09[2]	2228.26
P22	H-ENLKQNDISAEFTYQTKDA-NH_2	15-M05K[2]	2213.06
P23	H-YMMPVNSEV-NH_2	NUF2-003[2]	1067.48
P24	H-TNDVKTLADLNGVIEEEFT-NH_2	16-M09[2]	2106.05
P25	H-SAWLFRMWYIFDHNYLKPL-NH_2	16-M06[2]	2498.27

Supporting Peptides (**SP**) were just used for information documented in the supporting information and not in the main text. Their synthesis is described in the respective chapter.

*Synthesis of **P1-P4***

Peptides **P1**-**P4** were synthesized by Bachem UK (St. Helens) at 600 µmol scale on pre-loaded 2-chlorotrityl resins using a Gyros Protein Technology Symphony automated peptide synthesizer. All SPPS resins, Fmoc-amino acid derivatives and activators used were Bachem products. Solvents were purchased from Brenntag, Romil and Sigma-Aldrich. After synthesis, 300 µmol of each peptidyl-resin was sent to Belyntic GmbH for PEC purification with the different linkers as described in S3, and 300 µmol of peptidyl resin was retained at Bachem UK. After PEC purification, peptide samples were sent to Bachem UK to confirm the analysis performed by Belyntic.

*Synthesis of **P5***

The peptide **P5** (**Table S 1**) was synthesized in a 100 µmol scale on Rink Amide AM Polystyrene resin with single coupling of amino acid building blocks. Each coupling cycle was 60 min with 0.5 M amino acid (3 eq), 0.5 M Oxyma (3 eq) and 1 M DIC (4 eq) in DMF. At the end of each amino acid coupling, capping was performed with 2 M Ac_2O and 2 M pyridine in DMF (1:1) for 5 min. Each amino acid coupling preceded Fmoc-removal with 20% piperidine in DMF for 5 min (2x).

*Synthesis of peptides **P6-P25***

The peptides **P6-P25** (**Table S 1**) were synthesized in a 50 µmol scale on Rink Amide AM Polystyrene resin with single coupling up to amino acid 13 and double coupling for more than 13 amino acids. Each coupling cycle was 60 min with 0.5 M amino acid (3 eq), 0.5 M Oxyma (3 eq) and 1 M DIC (4 eq) in DMF. At the end of each amino acid coupling, capping was performed with 2 M Ac_2O and 2 M pyridine in DMF (1:1) for 5 min. Each amino acid coupling preceded Fmoc-removal with 20% piperidine in DMF for 5 min (2x).

S3 Comparative study of reductively cleavable linkers 2a, 2b, 2c with base-labile linker 1a

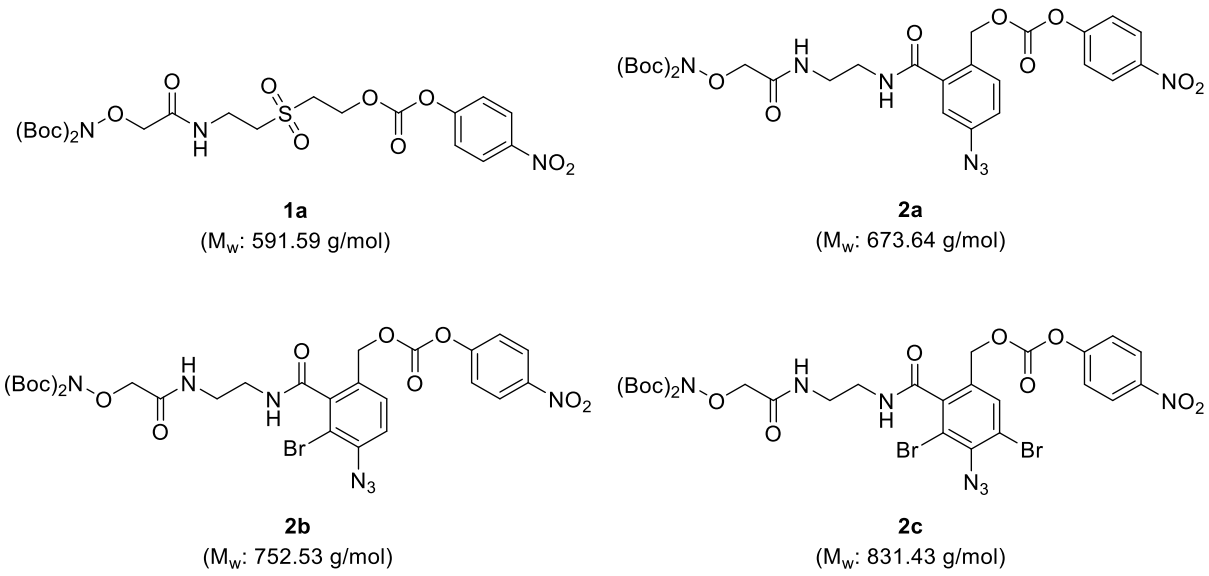


Figure S 1: Chemical structures of linker molecules **1a**, **2a**, **2b**, and **2c** used for the evaluation of c&r peptide purification.

Peptides **P1**-**P4** were synthesized by Bachem UK (St. Helens) as described in S2.4 on page 17 and send to Belyntic for the PEC purification.

S3.1 Crude purity determination

An aliquot of resin (~1 mg) was taken, and peptide was released by treatment with 100 µL Reagent K (TFA/H_2O/Phenol/EDT/Thioanisol; 82.5:5:5:2.5:5) for 2 h. Thereafter the peptides were precipitated by addition of 1 mL chilled Et_2O. and the samples were centrifuged and the pellets were washed once with Et_2O. After discarding the ether phase the pellets were dried on air and peptides dissolved in MeCN/water (3:7; 0.1% TFA). The resulting solution was analysed by UPLC-MS (gradient 00-60% B in 0-11 min, 90% B from 11-13 min). The resulting peaks with an absorption at 210 nm were integrated and the relative amount in percent of the peak-area of the desired peptide, as indicated by the corresponding ESI-MS mass at the same retention time as the peak, can be found in Table 2 (Main Text).

S3.2 Coupling of the linkers onto the peptides

In the following, the procedures for coupling of each linker to 50 µmol peptide (synthesis scale) is described. The individual coupling procedures were applied for the coupling to all peptides **P1**, **P2**, **P3** and **P4** (**Table S 1**, page 17). Of note, for linker **2b** of each of the four peptides two 50 µmol aliquots were taken and linker was coupled equally to the eight aliquots (four times for each peptide for PEC procedure with PPh_3 and four times with DTT as reducing agent).

S3.2.1 Coupling of linker 1a:

50 µmol peptide on SPPS resin was treated with DMF in a fritted SPPS cartridge for 15 min and the solvent was removed. 140.5 mg (237.5 µmol, 4.75 eq) linker **1a** and 60.5 mg (425 µmol, 8.5 eq) Oxyma were dissolved in 665 µL DMF and 73.5 µL (425 µmol, 8.5 eq) DIPEA was added. The solution was added to the cartridge and the mixture was shaken at rt for 2 h. The solution was removed by vacuum suction and the residue was washed 3x with 1 mL DMF and 3x with 1 mL DCM.

S3.2.2 Coupling of linker 2a:

50 µmol peptide on SPPS resin was treated with DMF in a fritted SPPS cartridge for 15 min and the solvent was removed. 134.7 mg (200 µmol, 4 eq) linker **2a** and 43 mg (300 µmol, 6 eq) Oxyma were dissolved in 500 µL DMF and 52.5 µL (300 µmol, 6 eq) DIPEA was added. The solution was added to the cartridge and the mixture was shaken at rt for 2 h. The solution was removed by vacuum suction and the residue was washed 3x with 1 mL DMF and 3x with 1 mL DCM.

S3.2.3 Coupling of linker 2b:

50 µmol peptide on SPPS resin was treated with DMF in a fritted SPPS cartridge for 15 min and the solvent was removed. 150.5 mg (200 µmol, 4 eq) linker **2b** and 43 mg (300 µmol, 6 eq) Oxyma were dissolved in 500 µL DMF and 52.5 µL (300 µmol, 6 eq) DIPEA was added. The solution was added to the cartridge and the mixture was shaken at rt for 2 h. The solution was removed by vacuum suction and the residue was washed 3x with 1 mL DMF and 3x with 1 mL DCM.

S3.2.4 Coupling of linker 2c:

50 µmol peptide on SPPS resin was treated with DMF in a fritted SPPS cartridge for 15 min and the solvent was removed. 166.3 mg (200 µmol, 4 eq) linker **2c** and 43 mg (300 µmol, 6 eq) Oxyma were dissolved in 500 µL DMF and 52.5 µL (200 µmol, 6 eq) DIPEA was added. The solution was added to the cartridge and the mixture was shaken at rt for 2 h. The solution was removed by vacuum suction and the residue was washed 3x with 1 mL DMF and 3x with 1 mL DCM.

S3.3 Cleavage of all linker-coupled peptides from SPPS resin

5 mL Reagent K (TFA/H_2O/phenol/EDT/thioanisol 82.5:5:5:2.5:5) was added to the air-dried linker-coupled peptides in the cartridges (50 µmol/sample) and the mixtures were shaken at rt for 2 h. The cleavage solutions were eluted into 50 mL centrifuge tubes and ~45 mL chilled diethyl ether was added to precipitate the crude linker-coupled peptides. After centrifugation, the supernatant was removed, and the residual pellets were washed once with diethyl ether. The solvent was decanted. The air-dried pellets were dissolved in MeCN/water (3:7; 0.1% TFA) and lyophilized overnight.

S3.4 Immobilisation of the linker-coupled peptides onto solid support

The following description was used for the immobilisation of 50 µmol (synthesis scale) of the individual linker-coupled peptides.

1.5 mL of modified agarose slurry was transferred into a 10 mL fritted cartridge and the solvent was removed by vacuum suction. The solid support (agarose) was washed 3x with water and 3x with 0.1 M aq. citric acid buffer (pH 4.5). The lyophilized crude linker-coupled peptide (50 µmol synthesis scale) was dissolved in 2.25 mL DMSO and 0.25 mL 0.1 M citric acid/7 M GdmCl buffer (pH 3.5) was added. The solution was added to the agarose beads and the mixture was shaken at rt for 2 h. At the beginning and at the end of the immobilisation, a 5 µL aliquot of the solution was diluted and analysed by UPLC-MS (gradient 0-70% B in 0-5 min, 90% B from 5-6 min) to check the rate of completion of the immobilisation. The solution was removed by vacuum suction and 2.5 mL of 2 w% L-cysteine in 0.1 M citric acid buffer (pH 4.5) was added. After shaking for 15 min, the solution was eluted. The agarose beads were washed 3x with 2.5 mL 0.9 M GdmCl in DMSO and 3x with 2.5 mL 0.1 M aq. NaCl/EtOH 3:7.

S3.4.1 Improved Recovery After Blocking with L-Cysteine:

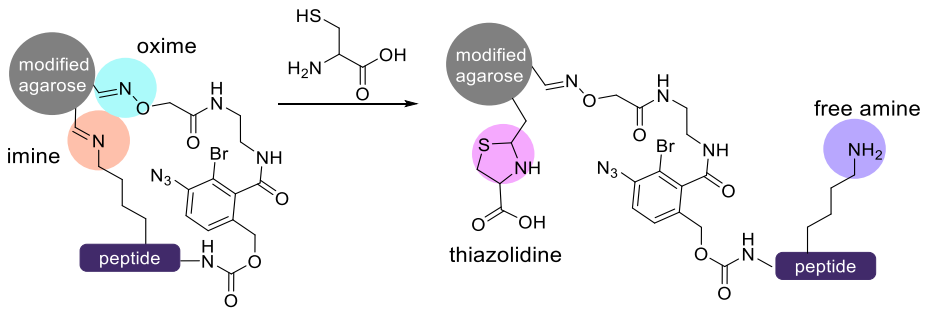
During first evaluations using reductively cleavable linker 2a and 2b with peptides **P4** and **P5** compared to peptide **SP2** (synthesis see S4.2 on page 33) it seemed, that peptides with lysine side chains have lower recovery then peptides without lysine side chains (see **Table S 2**).

Table S 2: Comparison of recoveries of the PEC process with PPh_3 using linker **2b** with peptides **P4**, **P5**, **SP1** and **SP2** with and without cysteine blocking.

number	sequence	origin	method	recovery[a]
P4	H-ARTKQTARKSTGGKA-OH	Histone H3 (1-15)	PPh_3 cleavage	33%
			cysteine blocking and PPh_3 cleavage	58%
P5	H-AKADEVSLHKWYG-NH_2	research peptide	PPh_3 cleavage	74%
			cysteine blocking and PPh_3 cleavage	90%
SP2	H-PSNPFYEALST-NH_2	LMTK3 (510-520)	PPh_3 cleavage	89%
			cysteine blocking and PPh_3 cleavage	100%

[a] recovery was calculated with equation S1 as described in section S3.6 on page 24.

For example, **P4** had a recovery of 33% and **P5** of 74% after the PEC purification. What was especially unsatisfactory in case of **P4**, whereas **SP2** had a recovery of 89%. This led us to the assumption, that imine formation may took place as shown in **Scheme S 4**. Especially since the agarose is functionalized in a way that neighbouring aldehyde groups are likely. Therefore, we incorporated a blocking step, where H-Cys-OH was added in buffer (pH 4.5) after peptide immobilisation, to form thiazolidine with excess aldehyde and also replace formed imines.[3]



Scheme S 4: Displacement of formed imines and formation of thiazolidine upon addition of cysteine.

The addition of cysteine after immobilisation led to an increase in recovery from 33% to 58% for **P4** and from 74% to 90% for **P5**. An increase in recovery was also observed for **SP2** from 89% to 100%.

Therefore, the cysteine capping was used in all c&r processes described here.

S3.5 Peptide release and elution

S3.5.1 Procedure for peptides coupled to linker 1a

1.125 mL 40% methylamine was diluted with water to a final volume of 50 mL (0.2 M). 0.312 mL AcOH was added (pH 10.9). 3 mL of this solution was added to the cartridge containing washed peptide (50 µmol synthesis scale) on agarose. The mixture was shaken at rt for 60 min and the solution was eluted into a 50 mL centrifuge tube. The agarose beads were washed 2x with 2.5 mL 0.1% TFA in MeCN/H_2O 3:7 and 2x with 2.5 mL 0.1% TFA in MeCN/H_2O 7:3. The combined solutions were lyophilized. The lyophilizate was dissolved in 30 mL water and lyophilized again. The lyophilizate was dissolved in 0.3 mL AcOH and diluted with 35 mL water. The purified peptide was obtained after another lyophilization of the solution.

S3.5.2 Procedure for peptides coupled to linker 2a, 2b, or 2c when PPh_3 was used for the linker reduction

250 mg PPh_3 was dissolved in 4.5 mL MeCN. 0.25 mL AcOH and 0.25 mL water was successively added. The solution was transferred into the cartridge containing 50 µmol (synthesis scale) of the respective peptide attached to agarose via linker **2a**, **2b**, or **2c**. The mixture was shaken for 15 min and the solution was removed by vacuum suction. The agarose beads were washed 3x with 2.5 mL MeCN/water 9:1, and 1 mL TFA/water 4:6 was added. After shaking at rt for 1 h, 1 mL TFA was added and the solution was eluted into a 50 mL centrifuge tube. The agarose beads were washed 2x with 1 mL TFA/water 95:5, which was then eluted into the same tube. ~45 mL chilled diethyl ether was added to precipitate the purified peptide. The mixture was centrifuged, and the solvent was decanted. The residual pellet was washed once with diethyl ether. The resulting precipitate was air-dried, dissolved in MeCN/water 1:1 and lyophilized.

S3.5.3 Procedure for peptides coupled to linker 2b and with the use of DTT for linker reduction

2.5 mL acetonitrile was added to 50 µmol (synthesis scale) washed linker-coupled peptide on agarose. 250 mg DTT was dissolved in 2.5 mL of 5 w% aq. $NaHCO_3$ (pH 8) and transferred into the cartridge. The mixture was shaken for 15 min and the solution was removed. The agarose beads were washed 3x with 2.5 mL water and 3x with 2.5 mL MeCN. 1 mL TFA/water 4:6 was added to each sample. After shaking at rt for 1 h, 1 mL TFA was added and the solution was eluted into a 50 mL centrifuge tube. The beads were washed twice with 1 mL TFA/water 95:5, which was eluted into the same tube. ~45 mL chilled diethyl ether was added to precipitate the

purified peptide. The mixture was centrifuged, the solvent decanted and the remaining pellet washed once with ether. The final residue was air-dried, dissolved in MeCN/water 1:1 and lyophilized.

Each lyophilized peptide sample was dissolved in 2.5 mL MeCN/water (3:7; 0.1% TFA). 5 µL of the solution was diluted 1/10 with 0.1% TFA in MeCN/H_2O 3:7. 50 µL of 0.1 mg/mL Fmoc-Glu-OH in H_2O/MeCN 17:3 (serving as internal standard) and 100 µL saturated aq. GdmCl was added. 5 µL of the resulting solution was analysed by UPLC-MS (gradient 0-70% B in 0-5 min, 90% B from 5-6 min). Samples with peptide **P4** were simply diluted in water with 0.1% TFA 1:40.

S3.6 Calculation of the recovery

Recovery in the contest of the presented c&r purification is the yield of purification, starting with the amount of peptide on SPPS-resin compared to the final amount of c&r purified peptide. Different ways for the calculation of the recovery of peptide after the purification process were analysed. It was found that, in comparison with optical methods, weighing of the material before and after the purification gives a reduced recovery, most likely since the peptide content of the crude material was not determined. We therefore choose the synthetic scale to represent the starting amount. To estimate the reduced amount due to uncomplete amino acid coupling the crude purity is multiplied with the scale, since the truncations can be seen well in the UV in the UPLC-chromatograms.

For calculation of the recovery Equation S1 (below) was used, where the crude purity was determined as described above in S3.1 on page 19 before linker coupling.

$$recovery\ (\%) = \frac{m_{final}\ (mg) \cdot final\ purity(\%)\ /\ MW_{peptide}\ (\frac{g}{mol})}{scale\ (mmol) \cdot \text{crude purity (\%)}} \cdot 100\ \% \qquad \text{equation S1}$$

S3.7 Results of the comparative linker study

The purities and recoveries of all peptides purified with different reductively cleavable linkers **2** and base-labile linker **1a** can be found in **Table S 3**: Results of PEC purification of peptides **P1-4** below. For Linker **2b** linker peptide conjugate **3b** reduction was performed and compared with PPh_3 and DTT.

Table S 3: Results of PEC purification of peptides **P1**-**4**

No.	crude purity	purity[a] after PEC using linker with cleavage stimulus					recovery[b] of PEC process using linker with cleavage stimulus				
		1a, NH_2Me	**2a**, PPh_3	**2b**, PPh_3	**2b**, DTT	**2c**, PPh_3	**1a**, NH_2Me	**2a**, PPh_3	**2b**, PPh_3	**2b**, DTT	**2c**, PPh_3
P1	67%	77%	91%	91%	96%	91%	74%	40%	74%	64%	44%
P2	42%	67%	83%	89%	90%	92%	60%	40%	70%	60%	42%
P3	75%	84%	88%	87%	92%	88%	46%	23%	56%	54%	32%
P4	36%	92%	89%	96%	91%	91%[c]	32%	22%	58%	59%	29%[c]

[a]purity determined by integration of UPLC-MS traces at 210 nm; [b] see above S3.6; [c]purity and recovery with dithiothreitol as reducing agent.

S3.7.1 Results of comparative purification of P1 using linker 1a, 2a, 2b and 2c

Peptide **P1** H-TRYQAKPVNRSTPISTGKEG-OH has been synthesized by SPPS as described in S2.4 on page 17 and purified as explained in this chapter in a 50 µmol scale. A summary of UV chromatograms of starting material, key intermediates and product of the PEC-process can be found below in **Figure S 2**. The purified peptide was lyophilized and gained as a white fluffy solid. In case of purification with **1a** using NH_2Me for linker cleavage 63.4 mg (21.7 µmol, 77% UV_{210nm} purity), in case of **2a** using PPh_3 for linker reduction 32.2 mg (10.6 µmol, 91% UV_{210nm} purity), in case of **2b** using PPh_3 for linker reduction 59.7 mg (19.7 µmol, 91% UV_{210nm} purity), in case of **2b** using DTT for linker reduction 48.9 mg (17.0 µmol, 96% UV_{210nm} purity) and in case of **2c** using PPh_3 for linker reduction 59.7 mg (19.7 µmol, 91% UV_{210nm} purity) was yielded. Main ESI-signals were in all cases corresponding to the calculated molecular weight of desired peptide product as shown in chapter S9.2 on page 76. UPLC-MS: t_R = 1.93 min (gradient 00-70% B in 0-5 min, 90% B from 5-6 min); ESI-MS calculated for $C_{93}H_{156}N_{30}O_{31}$ MW: 2189.16 Da, [m/z] MH^{2+} 1095.59, MH^{3+} 730.73, MH^{4+} 548.30; found: 1095.25, 730.76, 548.29); side product oxazolidinone: calculated +26.00 Da; found 26.02 from MH^{2+} 1095.25 → 1108.26.

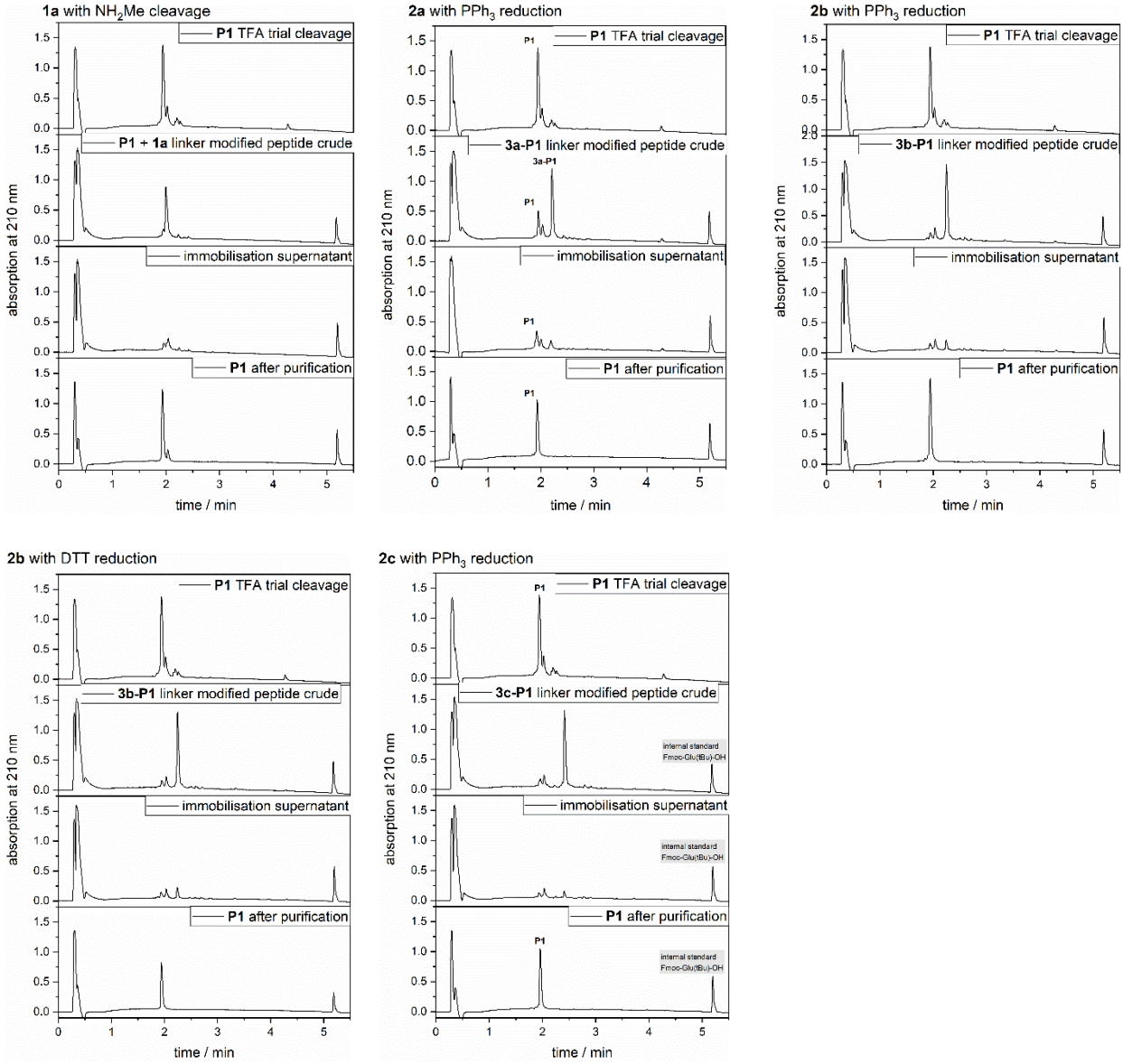


Figure S 2: Overview of UV chromatograms from the different steps of PEC purification of **P1** using linker **1a**, **2a**, **2b** with PPh_3 or DTT as reducing agent and **2c**. Peak at 5.2 min is internal standard Fmoc-Glu(*t*Bu)-OH.

S3.7.2 Results of comparative purification of P2 using linker 1a, 2a, 2b and 2c

Peptide **P2** H-RTGKLAPSFNGKSSQTREIL-OH has been synthesized by SPPS as described in S2.4 on page 17 and purified as explained in this chapter in a 50 µmol scale. An overview of UV chromatograms of starting material, key intermediates and product of the PEC-process can be found below in **Figure S 3**. The purified peptide was lyophilized and gained as a white fluffy solid. In case of purification with **1a** using NH_2Me for linker cleavage 41.4 mg (11.2 µmol, 67% UV_{210nm} purity), in case of **2a** using PPh_3 for linker reduction 22.3 mg (6.7 µmol, 83% UV_{210nm} purity), in case of **2b** using PPh_3 for linker reduction 36.0 mg (11.6 µmol, 89% UV_{210nm} purity), in case of **2b** using DTT for linker reduction 30.7 mg (10.0 µmol, 90% UV_{210nm} purity) and in case of **2c** using PPh_3 for linker reduction 21.2 mg (7.1 µmol, 92% UV_{210nm} purity) was yielded. UPLC-MS: t_R = 2.41 min (gradient 00-70% B in 0-5 min, 90%

B from 5-6 min); ESI-MS calculated for $C_{94}H_{160}N_{30}O_{30}$ MW: 2189.19 Da, [m/z] MH^{2+} 1095.60, MH^{3+} 730.74, MH^{4+} 548.31, found: 1095.60, 730.75, 548.69); side product -Arg^{17}-Glu^{18}-→ -Cit^{17}-Glu^{18}-: calculated +0.99 Da; found 1.60 from deconvoluted mass 2889.62 Da → 2191.22 Da.

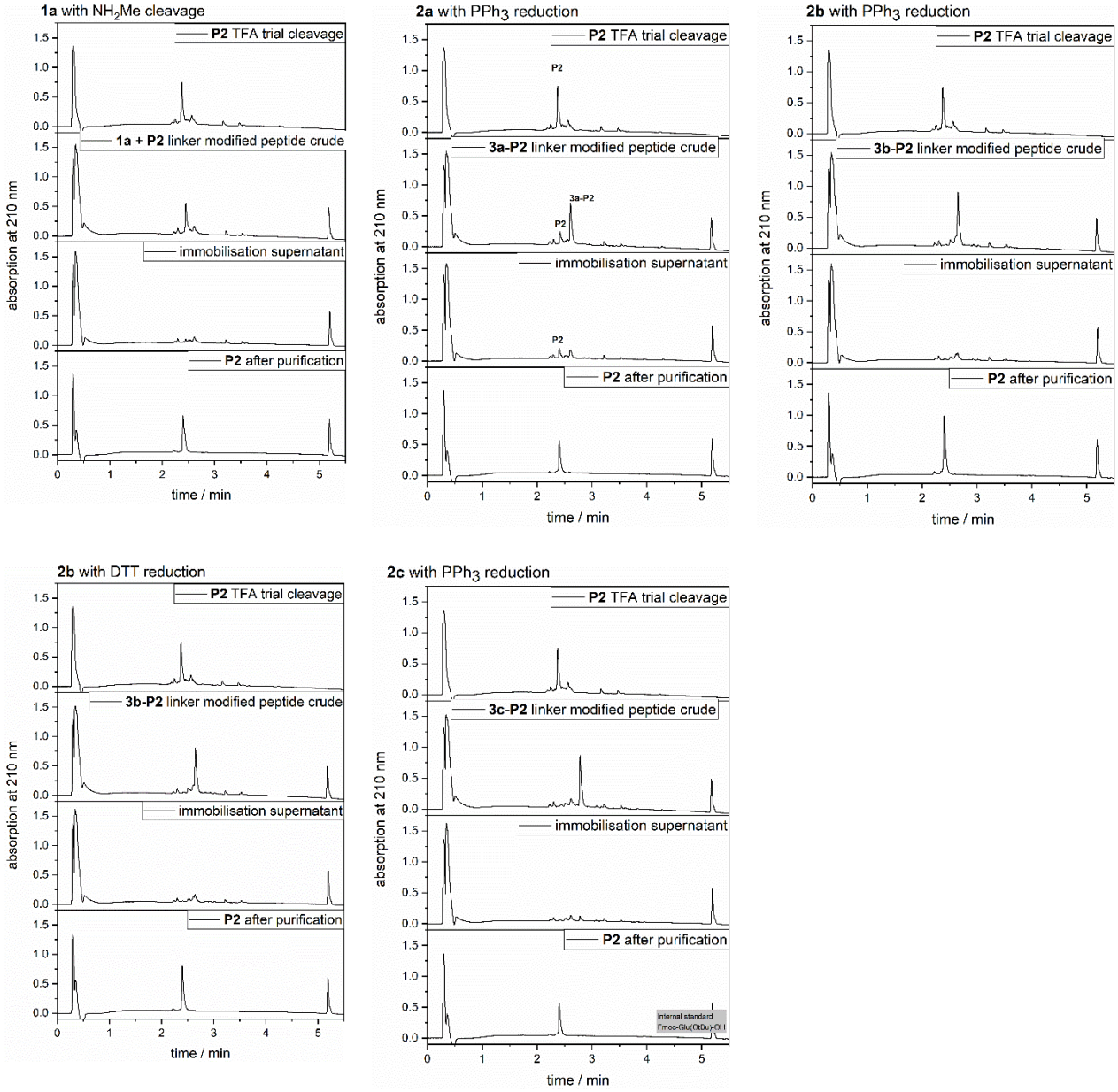


Figure S 3: Overview of UV chromatograms from the different steps of PEC purification of **P2** using linker **1a**, **2a**, **2b** with PPh_3 or DTT as reducing agent and **2c**. Peak at 5.2 min is internal standard Fmoc-Glu(*t*Bu)-OH.

S3.7.3 Results of comparative purification of P3 using linker 1a, 2a, 2b and 2c

Peptide **P3** H-DSAPNPVLDIDGEKLRTGTN-OH has been synthesized by SPPS as described in S2.4 on page 17 and purified as explained in this chapter in a 50 µmol scale. An overview of UV chromatograms of starting material, key intermediates and product of the PEC-process can be found below in **Figure S 4**. The purified peptide was lyophilized and gained as a white fluffy solid. In case of purification with **1a** using NH_2Me for linker cleavage 36.0 mg (13.2 µmol, 84% UV_{210nm} purity), in case of **2a** using PPh_3 for linker reduction 17.2 mg (6.2 µmol, 88% UV_{210nm} purity), in case of **2b** using PPh_3 for linker reduction 42.0 mg (14.9 µmol, 87% UV_{210nm} purity), in case of **2b** using DTT for linker reduction 38.7 mg (14.5 µmol, 92% UV_{210nm} purity) and in case of **2c** using PPh_3 for linker reduction 23.7 mg (8.51 µmol, 88% UV_{210nm} purity) was obtained. UPLC-MS: t_R = 2.53 min (gradient 00-70% B in 0-5 min, 90% B from 5-6 min); ESI-MS calculated for $C_{88}H_{146}N_{26}O_{34}$ MW: 2111.05 Da, [m/z] MH^{2+} 1056.53, MH^{3+} 704.69; found: 1057.02, 705.13); side product aspartimide: calculated -18.02 Da; found -18.45 deconvoluted mass from 2122.22 Da → 2093.77 Da.

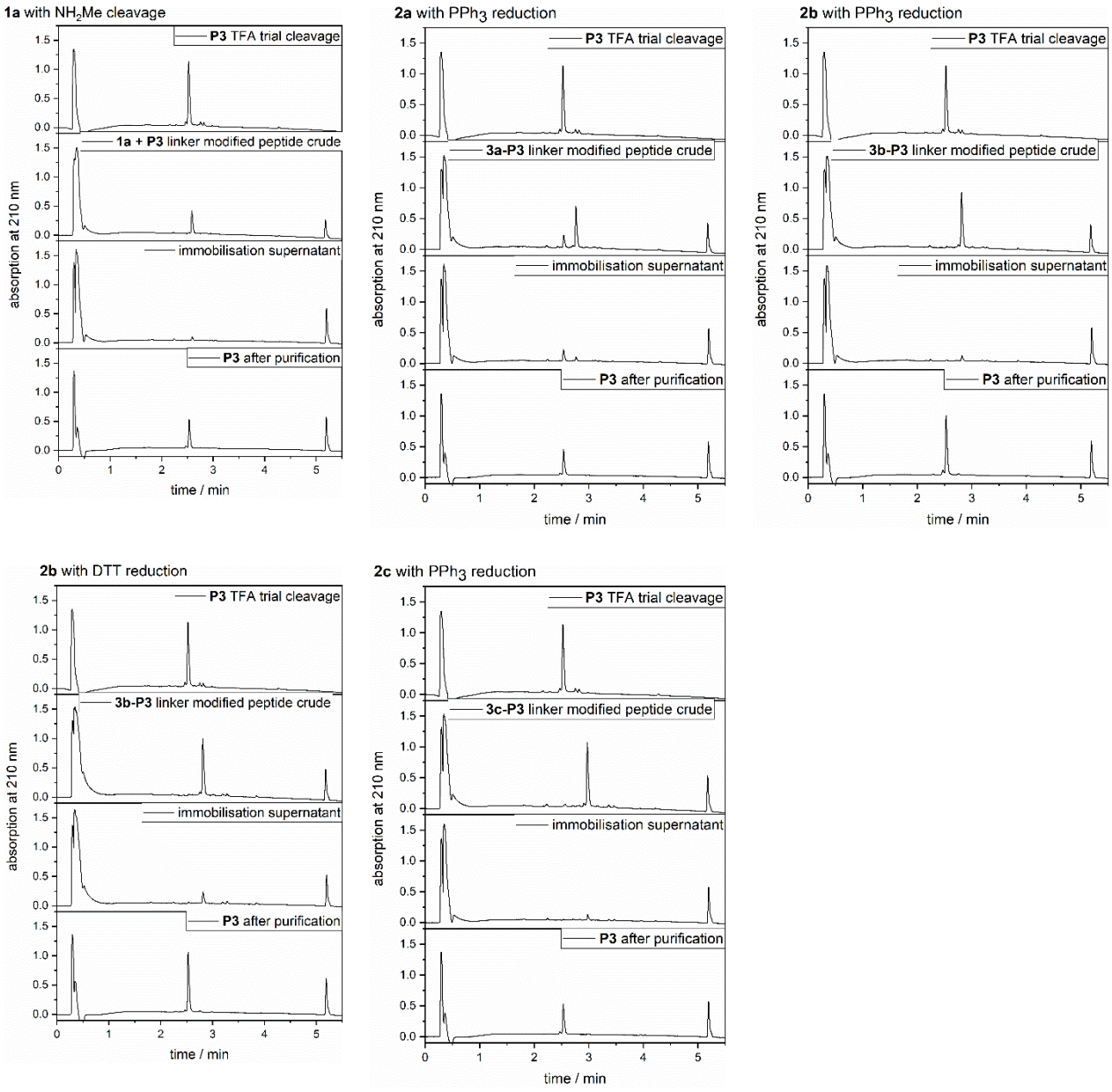


Figure S 4: Overview of UV chromatograms from the different steps of PEC purification of **P3** using linker **1a**, **2a**, **2b** with PPh_3 or DTT as reducing agent and **2c**. Peak at 5.2 min is internal standard Fmoc-Glu(*t*Bu)-OH.

S3.7.4 Results of comparative purification of P4 using linker 1a, 2a, 2b and 2c

Peptide **P4** H-ARTKQTARKSTGGKA-OH has been synthesized by SPPS as described in S2.4 on page 17 and purified as explained in this chapter in a 50 µmol scale. An overview of UV chromatograms of starting material, key intermediates and product of the PEC-process can be found below in **Figure S 5**. The purified peptide was lyophilized and gained as a white fluffy solid. In case of purification with **1a** using NH_2Me for linker cleavage 9.6 mg (4.6 µmol, 92% UV_{210nm} purity), in case of **2a** using PPh_3 for linker reduction 6.8 mg (2.7 µmol, 89% UV_{210nm} purity), in case of **2b** using PPh_3 for linker reduction 16.9 mg (7.2 µmol, 96% UV_{210nm} purity), in case of **2b** using

PPh_3 for linker reduction 18.3 mg (7.42 µmol, 91% UV_{210nm} purity) and in case of **2c** using DTT for linker reduction 10.6 mg (3.7 µmol, 78% UV_{210nm} purity) was yielded. UPLC-MS: t_R = 1.43 min (gradient 00-70% B in 0-5 min, 90% B from 5-6 min); ESI-MS calculated for $C_{63}H_{117}N_{25}O_{21}$ MW: 1559.89 Da[m/z] MH^{2+}: 780.95, MH^{3+}: 520.97, MH^{4+}: 390.98; found: 780.87, 520.93, 390.84);

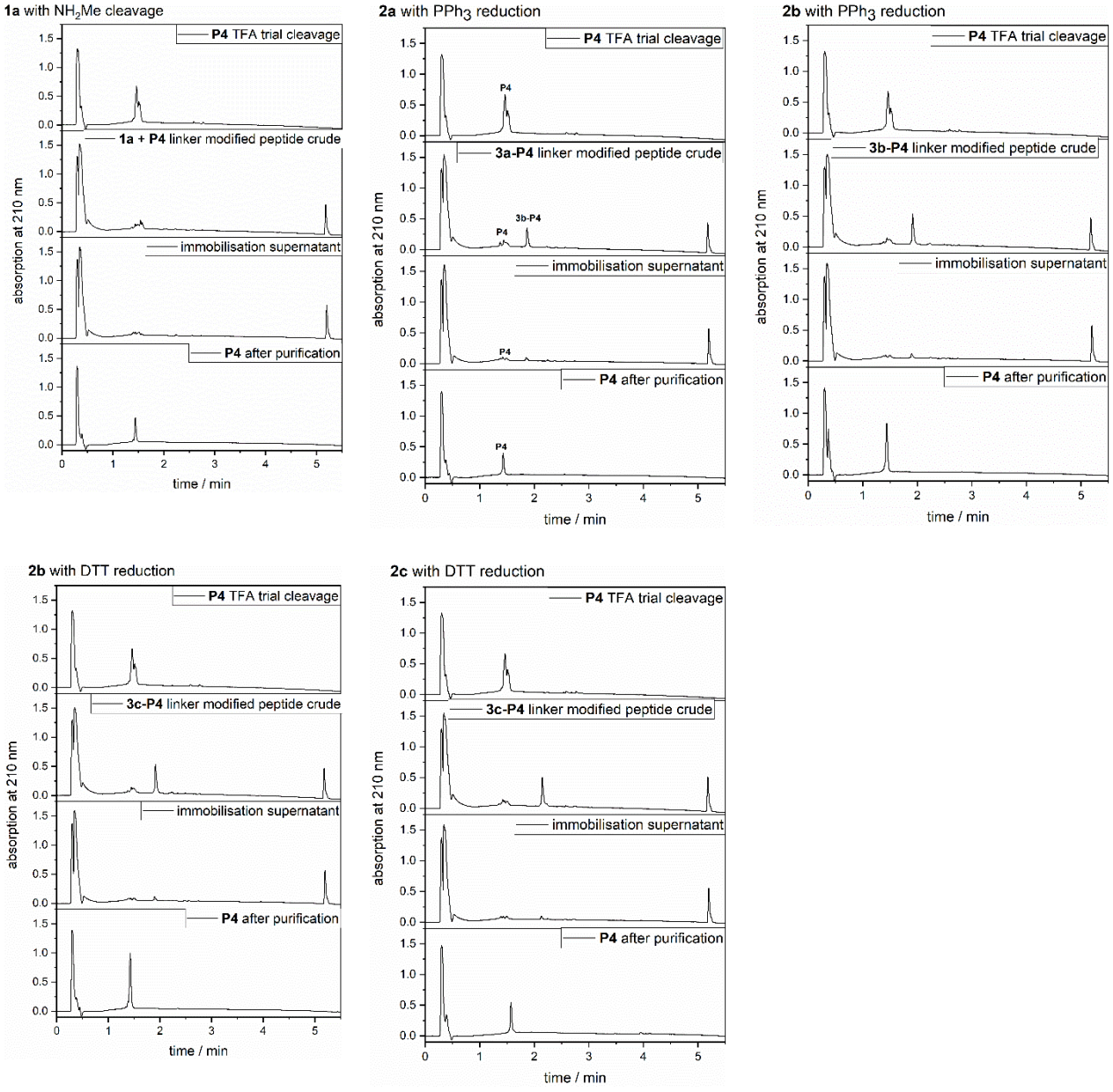


Figure S 5: Overview of UV chromatograms from the different steps of PEC purification of **P4** using linker **1a**, **2a**, **2b** with PPh_3 or DTT as reducing agent and **2c**. Peak at 5.2 min is internal standard Fmoc-Glu(*t*Bu)-OH.

S4 PEC evaluation studies

S4.1 Investigation of the pH-dependence on the immobilisation

Seven samples of 2.5 µmol linker-coupled peptide **3b-P5** (**3b**-AKADEVSLHKWYG-NH_2) were prepared by dissolving each in 135 µL DMSO. To the samples 15 µL of buffer solutions with different pH were added to gain a total volume of 150 µL. Starting point for buffer preparation was IB(GdmCl) (immobilisation buffer: 0.1 M citric acid with 7 M GdmCl, pH 3.5) which was used unaltered for one sample. For all other buffers, the pH was adjusted to 2, 3, 4, 4.5, 5 and 6 upon addition of anhydrous citric acid or anhydrous sodium citrate.

As reference for the starting point an eighth sample was prepared by dissolving 2.5 µmol linker-coupled peptide **3b-P5** in 150 µL DMSO/IB(GdmCl) (9:1). 10 µL of the solution was diluted 1:50 in MeCN/H_2O 3:7 with 0.1% TFA and 250 µL of Internal Standard solution (IS: 0.1 mg/mL Fmoc-L-Glu(OtBu)-OH in H_2O/MeCN 17:3 serving as internal UV standard in the UV chromatogram of UPLC-MS measurement) was added. The solution was analysed by UPLC-MS (gradient 00-70% B in 0-5 min, 90% B from5-6 min) and used for calculation of $\mathbf{Q_0}$ (equation S2) by dividing the AUC-value of the integral of linker-coupled **3b-P5** to that of the internal standard in the same chromatogram at 210 nm.

For the immobilisation of linker-coupled **3b-P5** from the individual solutions on the filter material, 7 fritted cartridges were filled each with 75 µL agarose beads suspension. The supernatant was removed, and the beads were washed 3x with water and 3x with 0.1 M citric acid buffer (pH 4.5). The peptide solutions were added to the cartridges and the mixtures were shaken at 800 rpm. After shaking for t = 15, 30, 60 and 90 min, a 10 µL aliquot of each sample was diluted and analysed by UPLC-MS as described above for the starting point reference sample.

For determination of the immobilisation efficiencies from the seven solutions over time, the AUC-values of the integrals of linker-coupled **3b-P5** at the chosen time intervals were divided by the AUC-values of the integrals of the internal standard signals in the same chromatograms at 210 nm. The immobilisation efficiencies were then calculated using equation S2.

$$Immobilization\ efficiency = \frac{1 - Q_x}{Q_0} \qquad \text{equation S2}$$

Q_x = ratio of the AUC-value of the integral of linker-coupled **3b-P5** to that of the internal standard at time point y, with y = 15, 30, 60, 90 min, Q_0 = ratio of the AUC-value of the integral of linker-coupled **3b-P5** to that of the internal standard at immobilisation starting point.

Table S 4: Immobilisation efficiencies of linker-coupled **3b-P5** in solutions at different pH over time. Immobilisation efficiencies were calculated using equation S2.

Time / min	**Immobilisation efficiency / %**						
	pH = 2	**pH = 3**	**pH = 3.5**	**pH = 4**	**pH = 4.5**	**pH = 5**	**pH = 6**
15	85	78	88	95	73	85	80
30	91	87	85	96	71	95	90
60	98	95	98	99	80	100	96
90	100	100	100	100	85	100	100

The resulting values (**Table S 4**) indicate quantitative immobilisation throughout the evaluated pH-range within 90 min. The lower immobilisation efficiency at pH 4.5 are most likely caused by pipetting errors.

However, slight differences for the immobilisation efficiency could be observed over the investigated pH-range by looking at the first timepoint, t = 15 min: Immobilisation efficiency increases with rising pH up to pH = 4, but decreases above pH = 4.5. Thus, between pH = 4 and pH = 5 a maximum for the immobilisation efficiency can be assumed (**Figure S 6**).

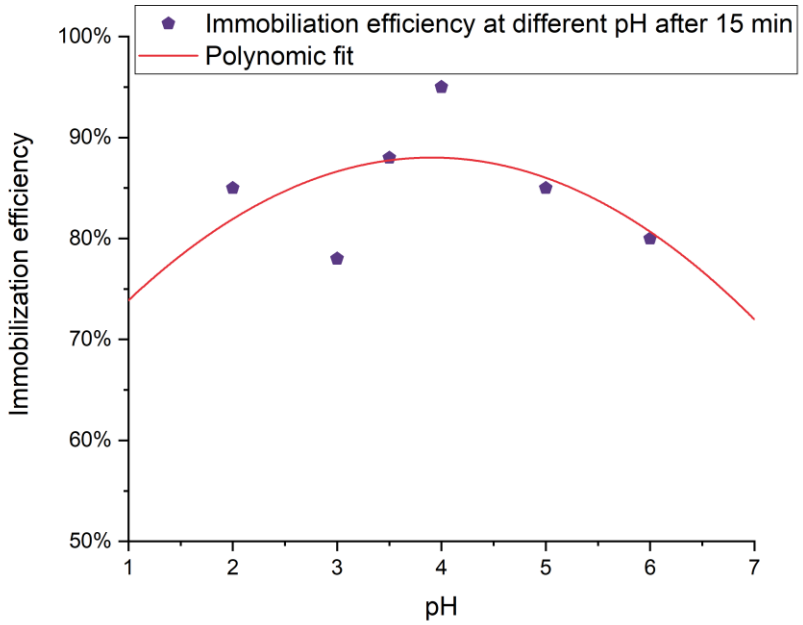


Figure S 6: Polynomic fit (second order) of the immobilisation efficiencies in dependence of the pH at t = 15 min (without the value for pH = 4.5).

S4.2 Influence of solvents and chaotropic additives on immobilisation

Table S 5. Number, sequence, origin and molecular weight (Mw) of peptides used in this immobilisation study.

number	Peptide	Origin	M_W (Da)	observed behaviour when dissolved in MeCN/H_2O (7:3)
SP1	H-YFTGSEVENVSVNVH-NH_2	CMV (81-95)	1678.80	insufficient dissolution and hydrogel formation
SP2	H-PSNPFYEALST-NH_2	LMTK3 (510-520)	1223.58	aggregation, poor immobilisation behaviour
SP3	H-DAEFRHDSGYEVHHQKLVFF-NH_2	amyloid-beta (1-20)	2459.18	insufficient dissolution and aggregation, hydrogel- and fibril-formation

To analyse the influence of solvents and chaotropic additives the three peptides **SP1**-**SP3** (**Table S 5**) were synthesized in 100 µmol scale as described for **P6**-**P25** in S2.4 on page 17. Linker **2b** was coupled as described in S3.2 on page 20 and TFA cleavage was performed with Reagent K as described in S3.3 on page 21. These peptides were chosen since they showed poor solubility and strong tendency to aggregate or form hydrogels (see **Table S 5**). One tenth of the total amount of peptides obtained after TFA cleavage, ether precipitation and lyophilisation was taken (10 µmol) and dissolved in 1 mL of solvents. The following solvent systems were compared: Hexafluoroisopropanol (HFIP), DMSO/IB (9:1) and DMSO/IB(GdmCl) (9:1). IB is the immobilisation buffer, a 0.1

M solution of sodium citrate adjusted with citric acid to pH 4.5. IB(GdmCl) is the same buffer with the addition of 7 M GdmCl, pH 3.5. Of note, HFIP did not dissolve linker-modified amyloid-beta (1-20) SP3 3b-SP3 completely and was found to have poor solubilising power for hydrophilic peptides. Each 300 µL agarose beads suspension (slurry) was used in fritted 2 mL syringe reactors and beads were conditioned as described in S3.4 on page 21. Before addition to the beads from each dissolved peptide an 10 µL aliquot was mixed with the internal standard and analysed by UPLC-MS as described in S4.1 on page 31. After different time intervals (30, 60, 90 min) immobilisation efficiencies were calculated like in S4.1 with equation S2, see **Figure S 7**.

HFIP gave the best results - all peptides were quantitatively immobilised after 90 min. Immobilisation from DMSO/IB(GdmCl) was slightly better than DMSO/IBbut both were not completed after 90 min.

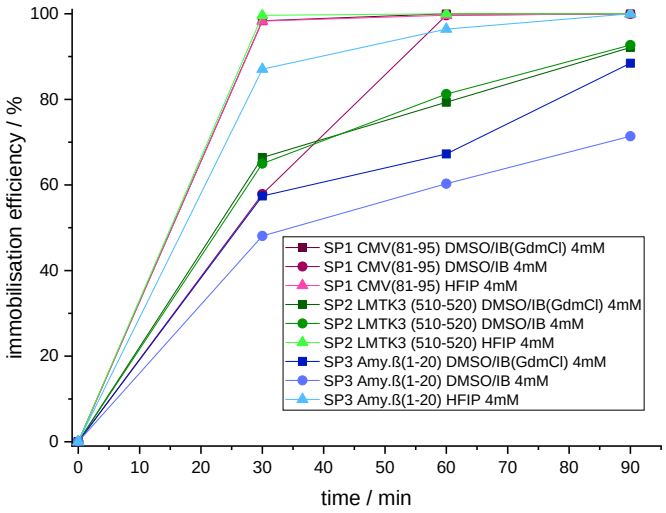


Figure S 7: Influence of different solvent systems on the immobilisation efficiency of difficult to solubilise and immobilise peptides.

Since DMSO showed in contrast to HFIP general applicability in dissolution of peptides (hydrophilic to hydrophobic) but insufficient immobilisation efficiencies, we used **SP3** amyloid-beta (1-20) to test if higher concentrations enable quantitative immobilisation within 90 min.

For that four samples of 4 µmol (each 1/10 of 100 µmol scale with a crude purity of 40%) of peptide **SP3** amyloid-beta (1-20) was dissolved in different volumes 320, 500, 1000 and 2000 µL of DMSO/IB(GdmCl) (9:1) to gain peptidic concentrations of 2, 4, 8 and 13 mM. The peptide aliquots were always dissolved in pure DMSO first and then 10 vol.% of the final volume of IB(GdmCl) was added. Thereafter the immobilisation efficiency was analysed as described above and is depicted in **Figure S 8**.

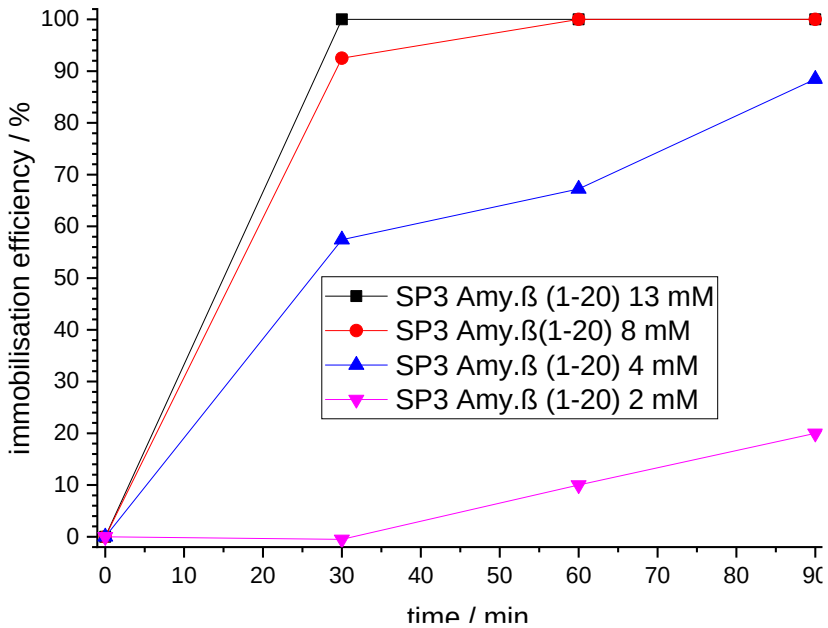


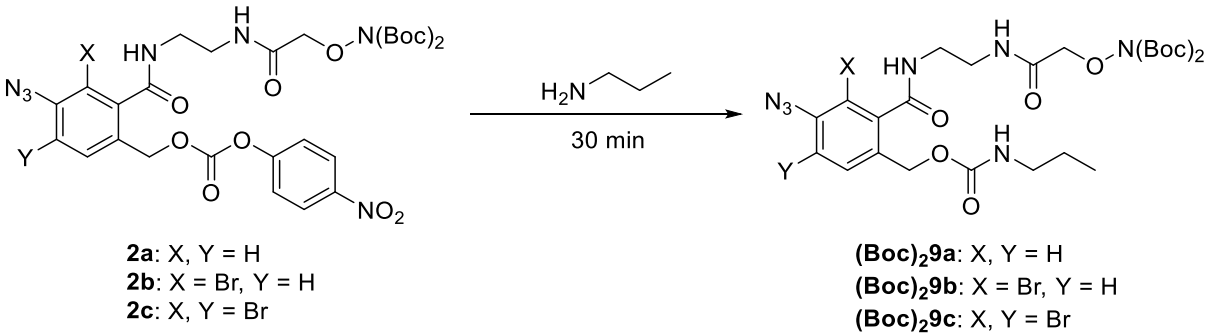
Figure S 8: Influence of different concentrations on the immobilisation efficiency of amyloid-beta (1-20) in DMSO/IB(GdmCl) 9:1.

A leap in the immobilisation efficiency was observed between a concentration of 4 mM and 8 mM. With a concentration of 8 or 13 mM, quantitative immobilisation was achieved within 90 min.

Therefore, it is recommended to use DMSO as a solvent for peptide dissolution since it dissolves a variety of different peptides from hydrophilic to hydrophobic peptide. The concentration of peptides should be 8 mM or higher.

S4.3 Evaluation of the linker stability with linker-propylamine carbamates

Procedures for the generation of linker-propylamine carbamates $(Boc)_2$9



2a: X, Y = H
2b: X = Br, Y = H
2c: X, Y = Br

$(Boc)_2$9a: X, Y = H
$(Boc)_2$9b: X = Br, Y = H
$(Boc)_2$9c: X, Y = Br

Scheme S 5: Reaction scheme for the generation of linker-N-propylamine-carbamates **$(Boc)_2$9**.

2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-N-propylamino-carbamate

(Boc)$_2$9a

According to **Scheme S 5**, 50 mg (74.3 µmol) linker **2a** was dissolved in 3 mL 1-propylamine. After stirring at room temperature for 30 min, excess propylamine was blown off by air stream. 30 mL of EtOAc was added and the organic layer was extracted 3x with 15 mL 5 w% aq. $NaHCO_3$ to remove para-nitrophenol, which was a residue from the last synthetic step in the linker production and now indicated by the yellow colour of the aqueous phase. The organic phase was dried over $MgSO_4$. After filtration, the organic solvent was evaporated under reduces pressure to yield 50 mg (74.3 µmol, quant.) of the title compound **(Boc)$_2$9a** as an amorphous yellow solid. UPLC-MS: t_R = 2.75 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); ESI-MS [m/z]: calculated [M-Boc]H^+: 494.24; found: 494.10.

2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-3-bromobenzyl-N-propylamino-carbamate ***(Boc)$_2$9b***

According to **Scheme S 5**, 500 mg (0.66 mmol) linker **2b** was dissolved in 5 mL 1-propylamine. After stirring at room temperature for 2 h, residual propylamine was removed by air stream. 30 mL of EtOAc was added and the organic layer was extracted 3x with 20 mL 5 w% aq. $NaHCO_3$ to remove para-nitrophenol, which was indicated by a yellow colour of the aqueous phase. The organic phase was dried over $MgSO_4$. After filtration, the organic solvent was evaporated under reduced pressure. The crude product was purified by column chromatography yielding 220 mg (0.33 mmol, 50%) compound **(Boc)$_2$9b** as a pale yellow solid. UPLC-MS: t_R = 2.62 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); ESI-MS [m/z]: calculated [M-Boc]Na^+: 594.06, 596.06; found: 594.13, 596.13.

2-((2-(2-bis-(tert-Butoxycarbonyl)-(aminooxy)acetamido)ethyl)amido)-4-azido-3,5-dibromobenzyl-N-propylamino-carbamate ***(Boc)$_2$9c***

70 mg (84.2 µmol) linker **2c** was dissolved in 3 mL 1-propylamine. After 30 min stirring at room temperature, excess propylamine was removed by air stream. 30 mL of EtOAc was added and the organic layer was extracted 3x with 15 mL 5 w% aq. $NaHCO_3$ solution to remove residual para-nitrophenol, which was indicated by a yellow colour of the aqueous phase. The organic phase was dried over $MgSO_4$. After filtration, the organic solvent was evaporated under reduced pressure to yield 70 mg (84.2 µmol, quant.) of the desired compound **(Boc)$_2$9c** as an

amorphous yellow solid. Analysis was performed after 1h treatment with TFA/TIS/H_2O 95:2.5:2.5, therefore the following data is for **9c**. UPLC-MS: t_R = 2.15 min (gradient 10-90% B in 0-3 min, 90% B from 3-4 min); ESI-MS [m/z]: calculated MH^+: 550.00, 552.00, 554.00; found: 549.87, 551.85, 553.76.

Testing of the TFA-stability

First, a sample of the substance **$(Boc)_2$9a** was analysed by UPLC-MS (gradient 10-90% B in 0-3 min, 90% B from 3-4 min).

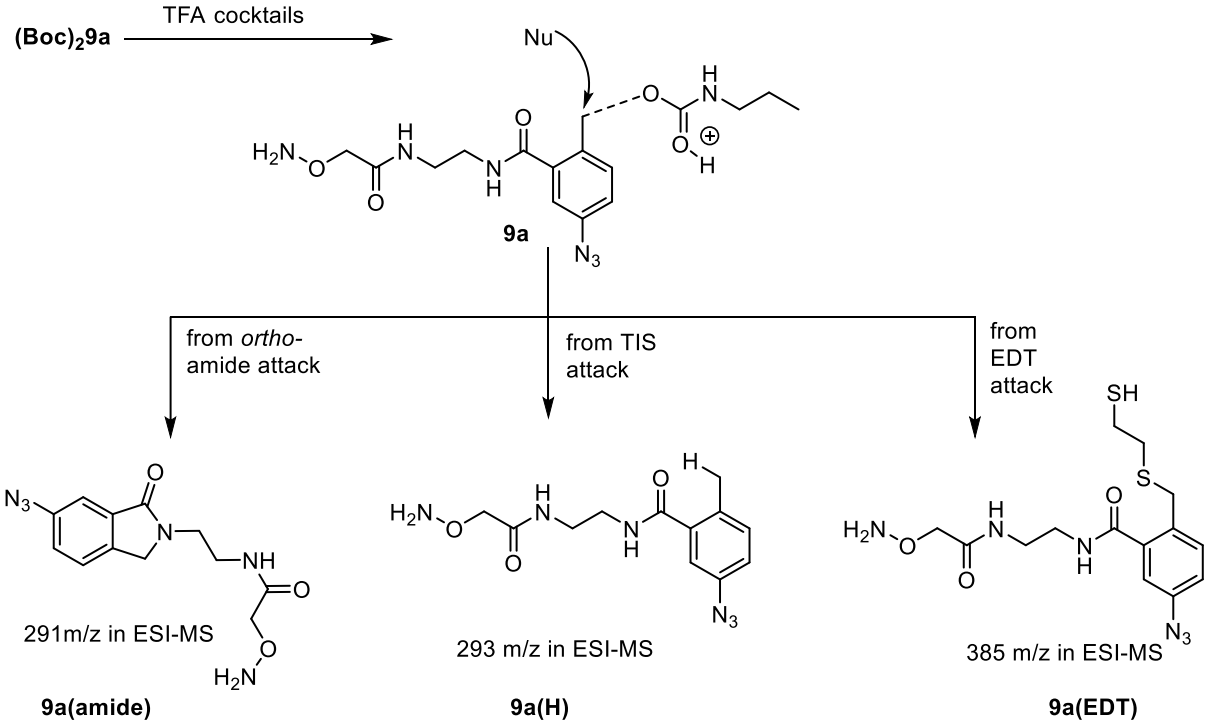
Then, 3x 1 mg -propylamine **$(Boc)_2$9a** was weighed in and dissolved in 0.1 mL of the following solutions:

A) Reagent K as described in S3.3 on page 21

B) TFA/H_2O/TIS, 95:2.55:2.5

C) TFA/H_2O/TIS, 97:2.5:0.5

The samples were shaken at rt. Immediately after dissolution and after t = 0.5 h, 1 h and 2 h, 5 µl aliquots of each sample were taken and diluted 1:20 in water. The sample were analysed by UPLC-UV-MS on column C2 (gradient 00-30% B in 0-3 min, 90% B from 3-4 min). The rising peaks were analysed by ESI-MS and three different degradation products could be identified as shown in **Scheme S 6**.



Scheme S 6: Degradation products generated during treatment of linker-N-propylamine-carbamate **$(Boc)_2$9a** with TFA-cleavage solutions.

The related amounts were judged by the peak integrals of the UV peaks at 278 nm showing the corresponding MS-signals of the species **9a(amide)**, **9a(H)** and **9a(EDT)**.

For **9a(H)**, no distinct peak could be detected in the UV chromatograms, but the corresponding mass was detected in the ESI-MS spectra. The resulting relative amounts of intact **9a** versus the degradation products over time in the three TFA solutions are shown below in **Figure S 9**.

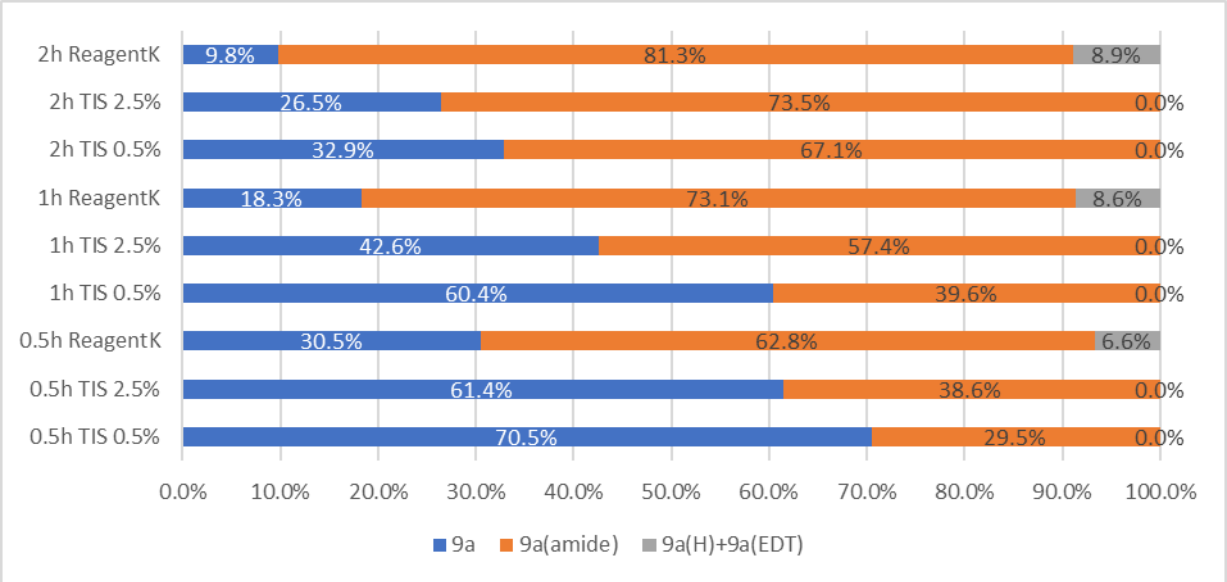


Figure S 9: Relative amounts of intact linker-propylamine conjugate **9a** relative to products **9a(amide), 9a(H)** and **9a(EDT)**resulting from nucleophilic attack; **9a(H)** was detectable in ESI-MS spectra but no UV signal could be detected at the corresponding t_R.

The linker-propylamine stability test also revealed a rate-enhancing influence of nucleophilic additives in the cleavage cocktail (Figure S1). Nucleophilic attack of thiols at the benzyl position might shift reaction equilibrium towards unmodified peptide as described by Bodanszky for the Cbz group.[4] Accordingly, the widely used TFA-cleavage cocktail Reagent K[5] with 5%(v/v) thioanisol and 2.5%(v/v) ethanedithiol (EDT) showed the fastest decomposition of **9a**. Likewise, a higher concentration of triisopropyl silane (TIS) in the TFA/TIS/H_2O cleavage cocktail accelerated the decomposition of **9a**, liberating propylamine as the decomposition product.

To compare the TFA stability of the three linkers **2a**, **2b**, and **2c**, the two linker-propylamine conjugates **$(Boc)_2$9b** and **$(Boc)_2$9c** were also evaluated in comparison to **$(Boc)_2$9a**. For this, 1 mg of each conjugate was dissolved in 0.1 mL Reagent K, 0.1 mLTFA/H_2O/TIS 95:2.5:0.5, or 0.1 mLTFA/H_2O/TIS 97:2.5:0.5. The samples were shaken at rt. Immediately after dissolution, after t = 30, 60, 120 min and for **$(Boc)_2$9b** and **$(Boc)_2$9c** also after t = 180 and 240 min, 5 µL of each solution was diluted 1:20 in water and analysed by UPLC-MS (gradient 10-90% B in 0-3 min, 90% B from 3-4 min). To ensure a constant amount of injection, remaining para-nitrophenol (resulting from

synthesis of linker-propylamine **9**) in the resulting chromatograms of all samples was used as internal standard. In each chromatogram (278 nm), all peaks in the range between 1.3 and 6.6 min were integrated and summed. Only peaks from para-nitrophenol and, if present, from EDT and phenol were excluded. The AUC-values of the integrated peaks of intact **9a**, **9b**, or **9c** were taken extra. With this, the relative amount of the linker-propylamine conjugates **9a**, **9b** and **9c** compared to that of decomposition products in percentage could be assessed over time. The results are summarised in **Table S 6**. The relative amount of each linker over time in the three TFA solutions is shown in **Figure S 10**. Based on the results, linker **2a** shows the lowest TFA-stability, while linker **2c** is the most stable one, though the stability of **2b** is also high, with >92% remaining intact after 120 min in Reagent K.

Table S 6: Stability of linker-propylamine conjugates **9a**, **9b** and **9c** in different TFA-cleavage cocktails. *UV_{278nm} integral of the peak of **9a**, **9b** and **9c** relative to the sum of increasing impurities. ** Sum of UV_{278nm} integrals of rising peaks of impurities relative to the peak of conjugate **9a**, **9b**, or **9c**.

Time / min	Reagent K			TFA/H_2O/TIS 95:2.5:2.5			TFA/H_2O/TIS 97:2.5:0.5		
	9a*	**9b***	**9c***	**9a***	**9b***	**9c***	**9a***	**9b***	**9c***
0	100%	100%	100%	100%	100%	100%	100%	100%	100%
30	31%	NA	NA	71%	NA	NA	61%	NA	NA
60	18%	95%	98%	60%	98%	98%	43%	98%	98%
120	10%	92%	97%	33%	96%	97%	27%	96%	97%
180	NA	88%	96%	NA	93%	96%	NA	94%	96%
240	NA	82%	NA	NA	91%	65%	NA	92%	NA
1440	NA	NA	69%	NA	NA	NA	NA	NA	84%
	Rising peaks of impurities**			**Rising peaks of impurities****			**Rising peaks of impurities****		
0	0%	0%	0%	0%	0%	0%	0%	0%	0%
30	70%	NA	NA	30%	NA	NA	39%	NA	NA
60	82%	5%	2%	40%	3%	2%	57%	2%	2%
120	90%	8%	3%	67%	4%	3%	74%	4%	2%
180	NA	12%	4%	NA	7%	4%	NA	6%	5%
240	NA	18%	NA	NA	9%	NA	NA	8%	NA
1440	NA	NA	31%	NA	NA	35%	NA	NA	16%

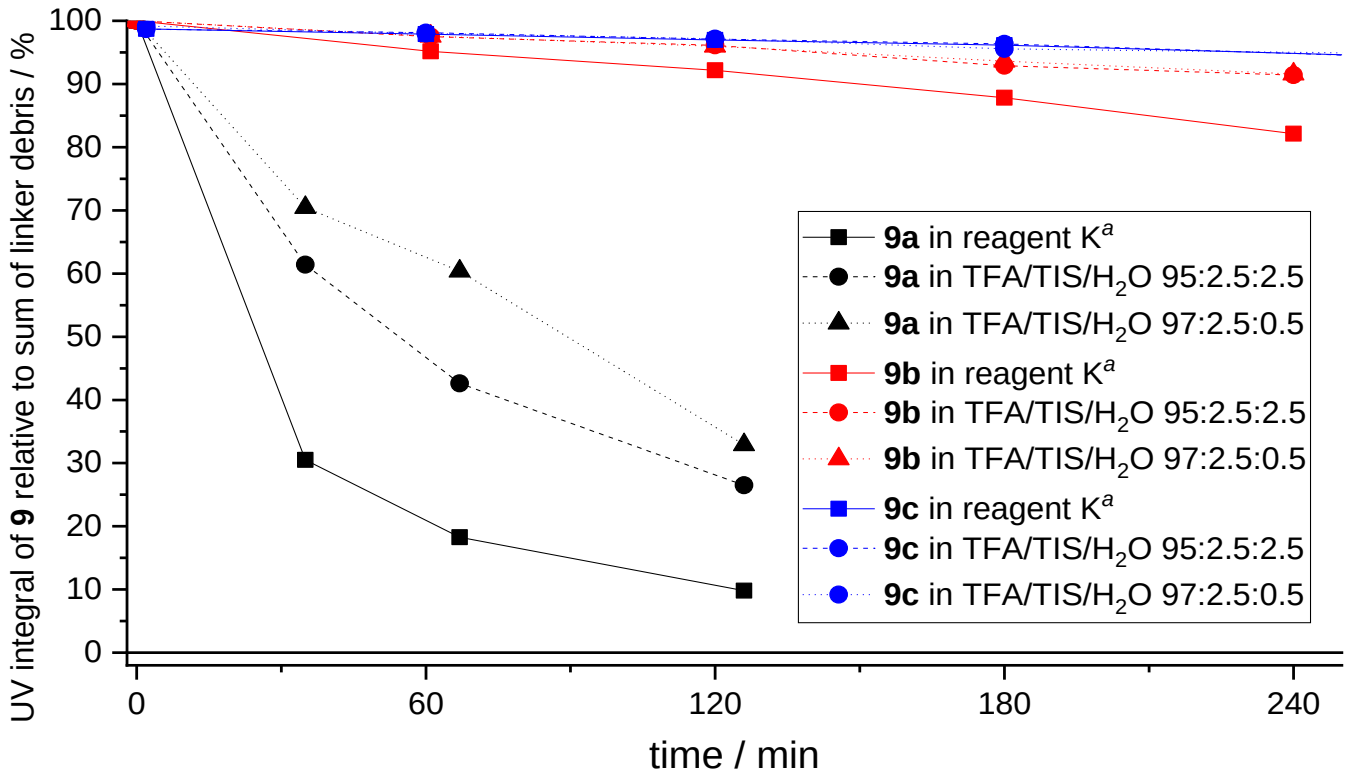
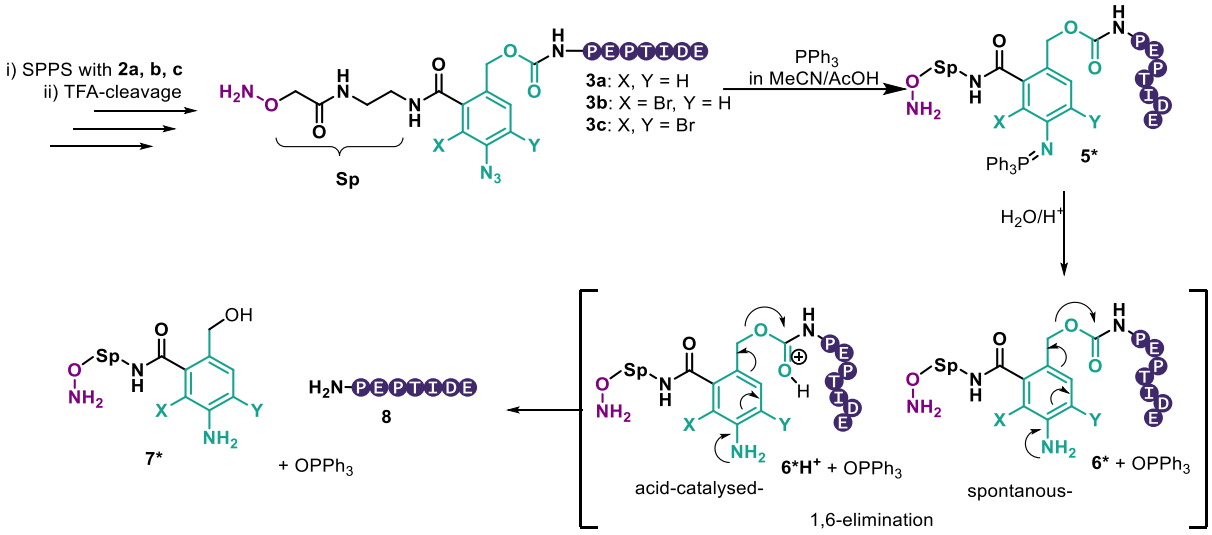


Figure S 10: Relative amounts of intact linker-propylamine conjugates **9a, 9b**, and **9c** over time in different TFA-cleavage cocktails.

S4.4 Evaluation of the azide reduction in linker conjugates 3 with PPh_3 in solution

To evaluate the reduction by PPh_3 of the different brominated azido-linker peptide conjugates **3** towards the native peptides **8** an investigation over time in solution of the involved species was conducted as depicted in **Scheme S 7**. The species were identified by UPLC-ESI-MS analysis after certain time intervals and their relative amounts were quantified by the integrals of the respective peaks in corresponding UV-chromatograms.



Scheme S 7: Assumed mechanism of PPh_3 reduction of linker-peptide conjugate **3** with subsequent spontaneous or acid-catalysed 1,6-elimination to yield the native peptide **8**.

The peptides used in this study are given below in **Table S 7**.

Table S 7. Number, sequence, origin and molecular weight (Mw) of peptides used in this study.

number	Peptide	Origin	M_W (Da)
SP4	H-CKADEVSLHKWYG-NH_2	research peptide	1533.74
SP5	H-TKADEVSLHKWYG-NH_2	research peptide	1531.78
P5	H-AKADEVSLHKWYG-NH_2	research peptide	1501.77

S4.4.1 Evaluation of reduction of non-bromo-linker peptide conjugate 3a-SP4

To **SP4** on Rink-amide PS resin was coupled **2a** as described in S3.2.2 on page 20 in 100 µmol scale. Thereafter TFA cleavage was performed with Reagent K as described in S3.3 on page 21. The linker tagged peptide **3a-SP4** was lyophilized.

From the lyophilized product **3a-SP4** 4 mg were dissolved in 200 µL MeCN and 50 µL H_2O. From this peptide stock for each reaction 33 µL corresponding to 0.44 µmol was taken. A stock solution of 57.7 mg PPh_3 in 1 mL MeCN/AcOH 9:1 was prepared. From this stock-solution aliquots were taken to dilute the PPh_3-stock to get the

following conditions: 50 eq. PPh_3 in MeCN, 20 eq. PPh_3 in MeCN, 10 eq. PPh_3 in MeCN, 20 eq. PPh_3 in MeCN/H_2O (9:1), 20 eq. PPh_3 in MeCN/AcOH (9:1) and 20 eq. PPh_3 in MeCN/H_2O/AcOH (8:1:1). 33 µL of peptide stock was mixed with 67 µL of each of the six different PPh_3 reduction cocktails. After 5, 15, 30 and 60 min 5 µL of reaction sample was withdrawn and diluted 1 to 10 with 45 µL of MeCN/H_2O 3:7 with 0.1% TFA and thereafter analysed by UPLC-MS (gradient 00-50% B in 0-3 min, 90% B from 3-4 min). The corresponding ESI MH^{2+} masses for linker-modified peptide **3a-SP4** 935 m/z and free peptide **SP4** 778 m/z were extracted and integrated. The relative AUC of free peptide **SP4** signal integration to linker-modified peptide **3a-SP4** signal integration were plotted against time and can be found in **Figure S 11**. The results lead to the conclusion that 20 eq of PPh_3 are required for complete reduction in 10-20 min and small amounts of water or acid slow down the reaction times. However, the differences are very small and after 30 min complete reduction was observed for all conditions.

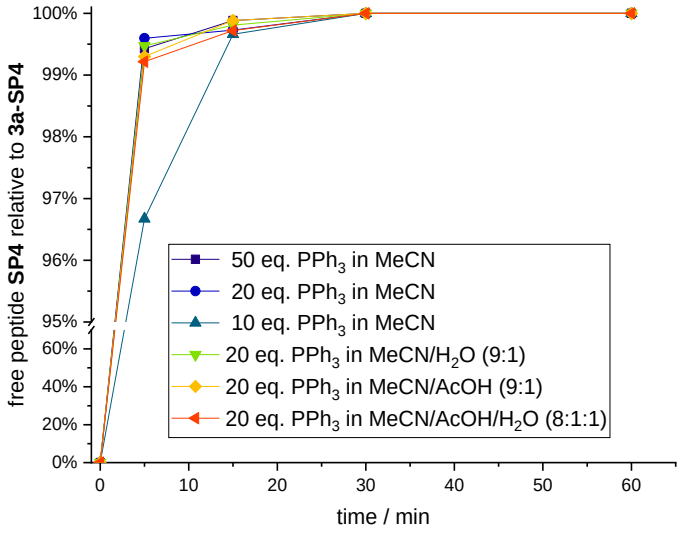


Figure S 11: Evaluation of necessary equivalents and solvent influence on the reduction using PPh_3 and elimination of linker modified peptide 3a-SP4.

S4.4.2 Evaluation of reduction of mono-bromo-linker peptide conjugate 3b-SP4

To **SP5** on Rink-amide PS resin was coupled **2b** as described in S3.2.3 on page 20 in 100 µmol scale. Thereafter TFA cleavage was performed with reagent K as described in S3.3 on page 21. The linker tagged peptide **3b-SP5** was lyophilized.

From the lyophilized product **3b-SP5** 3.3 mg were dissolved in 500 µL MeCN/AcOH/H_2O 8:1:1 and 83.3 µL of a 10 mg/mL PPh_3 solution in MeCN/AcOH (9:1) was added and shaken for 30 min. UPLC/MS analysis confirmed complete transformation towards the reduced linker-peptide conjugate **6*b-SP5** after 30 min. After 60 min, of this solution four 70 µL aliquots were transferred in 1.5 mL reaction vials. To these aliquots70 µL TFA/H_2O mixtures were added with TFA contents of 10, 20, 30 or 40% TFA leading to a total TFA content of 5, 10, 15 or

20% respectively. Each 30 min samples were taken and analysed on UPLC-MS. Peaks were assigned by ESI-MS and integrated, the relative amounts of 210 nm peak integrals of identified species are listed below in **Table S 8**.

Table S 8: Relative amounts of linker-modified peptide **3b-SP5**, the reduced linker-peptide conjugate **6b-SP5** or liberated peptide **SP5** in solutions of different TFA content

	time	amount of linker-modified peptide **3b-SP5** [a]	amount of reduced linker-peptide conjugate **6*b-SP5** [a]	free peptide **SP5** [a]
Staudinger reduction	30 min	0 %	85 %	15 %
	60 min	0%	62 %	38 %
Hydrolysis with 5 % TFA	30 min	-	37 %	63 %
	60 min	-	25 %	75 %
	90 min	-	20 %	80 %
Hydrolysis with 10 % TFA	30 min	-	26 %	74 %
	60 min	-	15 %	85 %
	90 min	-	< 5 % [b]	> 95% [b]
Hydrolysis with 15 % TFA	30 min	-	21 %	79 %
	60 min	-	15 %	85 %
	90 min	-	< 5% [b]	> 95 % [b]
Hydrolysis with 20 % TFA	30 min	-	23 %	77 %
	60 min	-	11 %	89 %
	90 min	-	< 5 % [b]	> 95 % [b]

[a] amounts determined by integration of UPLC-UV/vis chromatograms at 210nm; [b] due to overlapping signals peak integration only estimates are given

After 90 min in all TFA concentrations above 10% the peptide **SP5** was liberated from the reduced linker conjugate **6*b-SP5** to more than 95%.

S4.4.3 Evaluation of reduction of di-bromo-linker peptide conjugate 3c-P5

To **P5** on Rink-amide PS resin was coupled **2c** as described in S3.2.4 on page 21 in 100 µmol scale. Thereafter TFA cleavage was performed with reagent K as described in S3.3 on page 21. The linker tagged peptide **3c-P5** was lyophilized.

From the lyophilized product **3c-P5** 2.6 mg were dissolved in 500 µL MeCN/AcOH 9:1 and 83.3 µL of a 10 mg/mL PPh_3 solution in MeCN/AcOH (9:1) was added and shaken for 30 min. UPLC/MS analysis confirmed transformation towards the reduced linker-peptide conjugate **6*c-SP5** after 5 min. Of this solution six 70 µL aliquots were transferred to 1.5 mL reaction vials. To one aliquot 70 µL of TFA/H_2O (2:98) was added leading to a total TFA concentration of 1%. To the other five aliquots different volumes of pure TFA were added to have TFA concentrations of 22, 30, 42, 50 and 75%. After 30 min and 100 min samples were taken, diluted with

MeCN/H_2O 3:7 with 0.1% TFA and analysed on UPLC-MS. Peaks were assigned by ESI-MS analysis and integrated. The relative amounts of 210 nm peak integrals of identified species are listed below in **Table S 9**.

Table S 9: Relative amounts of linker-modified peptide **3c-P5**, the reduced linker-peptide conjugate **6c*-P5** or liberated peptide **P5** with solutions of different TFA content.

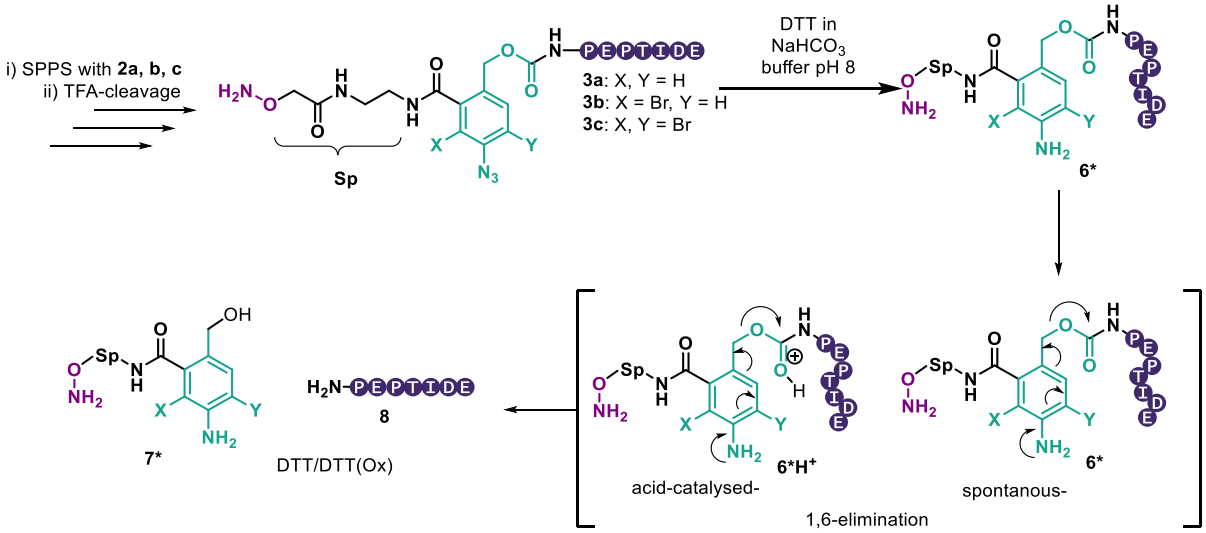
	time	amount of linker-modified peptide **3c-P5** [a]	amount of reduced linker-peptide conjugate **6*c-P5** [a]	free peptide **P5** [a]
Staudinger reduction	5 min	0 %	99 %	1 %
Hydrolysis with 1 % TFA	60 min	-	78 %	22 %
	24 h	-	54 %	46 %
Hydrolysis with 22 % TFA	30 min	-	65 %	35 %
	100 min	-	59 %	41 %
Hydrolysis with 30 % TFA	30 min	-	56 %	44 %
	100 min	-	32 %	58 %
Hydrolysis with 42 % TFA	30 min	-	44 %	56 %
	100 min	-	25 %	75 %
Hydrolysis with 50 % TFA	30 min		33 %	66 %
Hydrolysis with 75 % TFA	30 min		12 %	88 %

[a] amounts determined by integration of UPLC-UV/vis chromatograms at 210nm

In contrast to the evaluation of **6*b** modified peptide **6*b-SP5**, dibromo linker-conjugate **6*c** in **6*c-P5** needed higher amounts of TFA and longer times to liberate peptide **P5**. Higher concentrations of TFA yielded higher amounts of free peptide, but no quantitative linker hydrolysis was observed.

S4.5 Evaluation of the stability of linker conjugates 3a, 3b, and 3c after reduction with DTT in solution

To evaluate the reduction by DTT of the different brominated azido-linker peptide conjugates **3** towards the native peptides **8** an investigation in solution of the involved species was conducted as depicted in **Scheme S 8**. Relative amounts were determined by integrations of the respective peaks in UV-chromatograms at 210 nm after certain time intervals. The peaks were assigned to the corresponding species by ESI-MS.

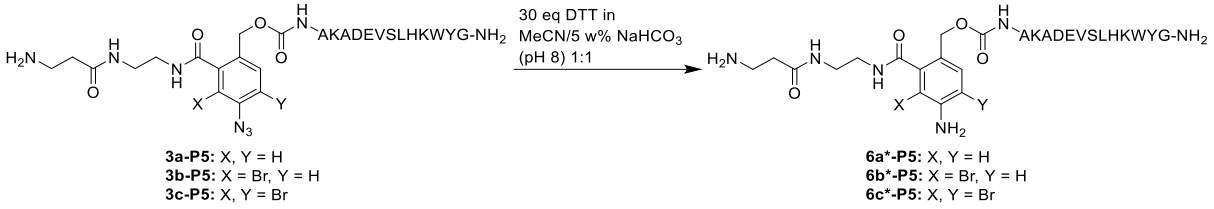


Scheme S 8: Assumed mechanism of DTT reduction of linker-peptide conjugate **3** with subsequent spontaneous or acid-catalysed 1,6-elimination to yield the native peptide **8**.

Linker coupling and TFA-cleavage

Three samples of 25 mg (5 µmol synthesis scale, 1 eq) sidechain protected H-AKADEVSLHKWYG-NH_2 **P5** on SPPS resin in 2 mL fritted cartridges were incubated with DMF for about 15 min and the solvent was removed. A solution of 13.5 mg (20 µmol, 4 eq) linker **2a,** 4.3 mg (30 µmol, 6 eq) Oxyma and 5.3 µL (30 µmol, 6 eq) DIPEA in 50 µL DMF was added to one sample, a solution of 15.1 mg (20 µmol, 4 eq) **2b**, 4.3 mg Oxyma and 5.3 µL DIPEA in 50 µL DMF to the second one, and a solution of 16.6 mg (20 µmol, 4 eq) **2c**, 4.3 mg Oxyma and 5.3 µL DIPEA in 50 µL DMF to the third one. The mixtures were shaken at rt for 2 h. The solvent was eluted, and the beads were washed 3x with DMF and 3x with DCM. After air-drying, 0.5 mL TFA/H_2O/DTT/TIS 91:4:3:2 was added to each sample and the mixtures were shaken at rt for 2 h. The solutions were filtered into 15 mL tubes and ~14 mL chilled diethyl ether was added to precipitate the peptides. The mixtures were centrifuged, and the supernatant was discarded. The residual pellets were washed once with ether and air-dried. The crude peptides were dissolved in MeCN/H_2O (1:1) and lyophilized.

Stability of the linker-conjugated peptides after reduction



Scheme S 9: Reduction of linker-coupled peptides with DTT in solution.

~5 mg (2.5 µmol) of each of the crude lyophilized linker-peptides **3a-P5**, **3b-P5**, and **3c-P5** was dissolved in 125 µL MeCN. 3x 12.5 mg (78 µmol, 30 eq) DDT was dissolved each in 125 µL 5 w% aq. $NaHCO_3$ (pH 8.16) and added to the peptide solutions. The samples were vortexed and 5 µL of each solution was diluted 1:40 in MeCN/H_2O (3:7 with 0.1% TFA) and analysed by UPLC-MS (gradient 00-70% B in 0-5 min,90% B from 5-6 min) as starting point (t = 0 min). The samples were kept at rt. At t = 15, 30, 45, 60, 120, 180, 240 min, another 5 µL aliquot of each sample was diluted and analysed by UPLC-MS as described above. Excerpts of the resulting UV chromatograms (210 nm) at t = 0, 15, 240 min of each sample are superimposed in **Figure S 12 A**, **B**, and **C**. Figure S3 **D** summarizes the ratio of free vs. linker-coupled peptide over time for the three conjugates. After 15 min, the azide group in each linker **2a**, **2b**, and **2c** was completely reduced. But after 2 h, the reaction showed different behaviour of the linker-peptide conjugates **6***: While conjugate **6*a** decomposed and released the free peptide **P5** almost entirely after 2 h, linker-conjugate **6*b** and **6*c** remain mainly intact and release only a small amount of free peptide.

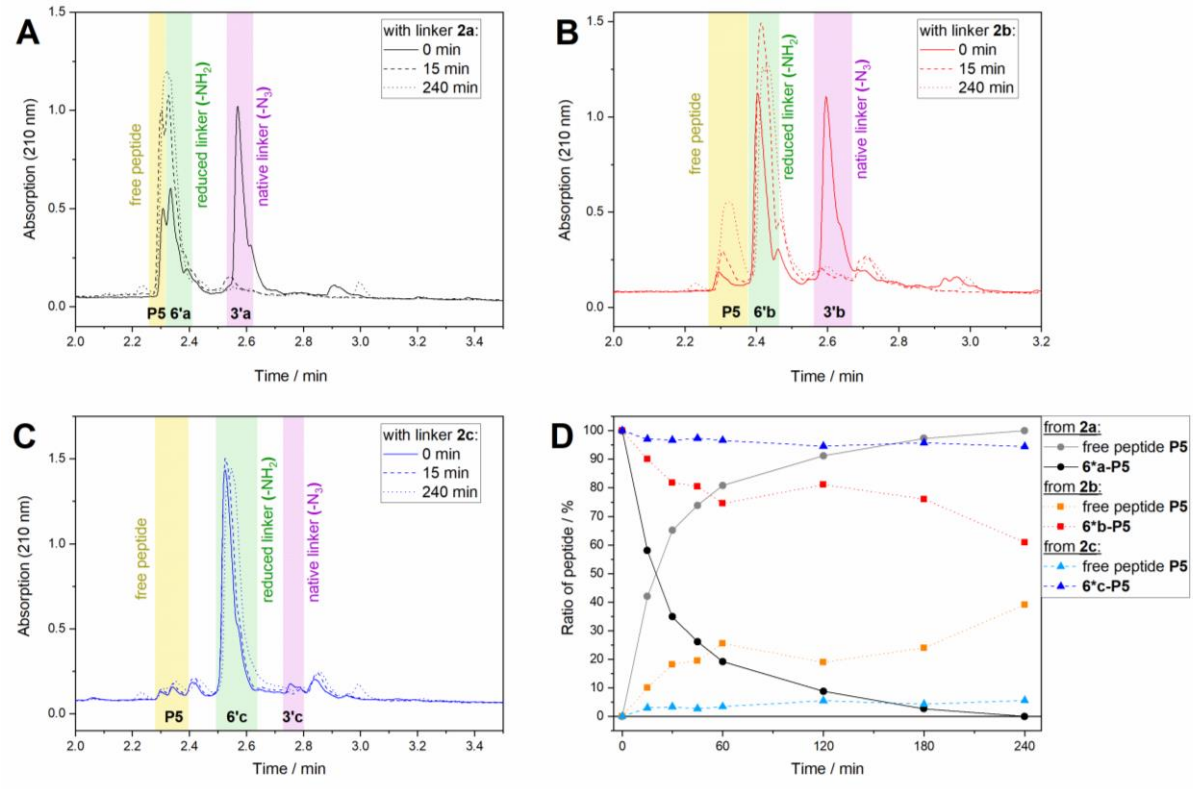


Figure S 12: Superimposed excerpts of UV chromatograms of linker-conjugate **6*a(P5) (A)**, **6*b(P5) (B)**, and **6*c(P5) (C)** at different time points in 0.3 M DTT in MeCN/0.6 M $NaHCO_3$ 1:1 at pH 8. **D)** Comparison of the ratio of free vs. linker-conjugate over time for peptide **P5** coupled to linker **2a**, **2b**, or **2c**. The amount of compound was determined by integration of the corresponding mass peaks extracted from the respective TIC chromatograms.

S4.6 Evaluation of the stability of 6*a-P5, 6*b-P5, 6*c-P5 at pH 8 after reduction with DTT on solid support and in 5% TFA solution

Linker-coupling and TFA-cleavage was carried out following the procedure described above in S4.4.

The other half (5 µmol of each linker-coupled peptide; 8.6 mg AP-L3.0, 9.7 mg AP-L3.2 and 10 mg AP-L3.3) was immobilised on agarose. For this, each peptide sample was dissolved in 0.225 ml DMSO and 25 µl buffer#2 (7 M GdmCl in buffer#1 (citric acid buffer at pH 4.5)) was added. Meanwhile, three 2 ml fritted syringe reactors were filled each with 0.15 ml modified agarose slurry. The supernatant was removed, and the beads were washed with water and buffer#1. The dissolved linker coupled peptides were added to the cartridges and the mixtures were shaken for 3 h at rt. The supernatants were analysed, and no linker-modified peptide could be detected, thus, suggesting complete immobilisation. The supernatants were removed and 0.25 ml 2 w% L-Cys in buffer#1 was added to each cartridge. After stirring for 15 min at rt, the solutions were removed, and the beads were washed with 0.9 M GdmCl in DMSO and 0.1 M aq. NaCl/EtOH (7:3). The samples were stored overnight in the fridge.

0.25 ml MeCN was added to each cartridge and 50 mg DTT dissolved in 0.25 ml 5 w% aq. $NaHCO_3$ was added. The mixtures were shaken for 4 h at rt. After t = 0, 15, 30, 45, 60, 120, 180, 240 min, 5 µl of the supernatant of

each of the 3 samples was diluted 1:20 with MeCN/H_2O (3:7) and thereafter 1:1 with IS and analysed by UPLC-MS (gradient 00-70% B in 0-5 min, 90% B from 5-6 min). The supernatant was eluted, and the beads were successively washed 3x with water and MeCN. 0.25 ml 5% TFA in water was added to each cartridge and the mixtures were shaken for 4 h at rt. After t = 0, 15, 30, 45, 60, 120, 180, 240 and 900 min, 5 µl of the supernatant of each of the 3 samples was diluted 1:20 with MeCN/H_2O (3:7) and thereafter 1:1 with IS and analysed by UPLC-MS (gradient 00-70% B in 0-5 min, 90% B from 5-6 min).

From all measured UV-chromatograms the peaks of liberated native peptide **P5** were integrated. **P5** integral out of DTT supernatant from the **6*a-P5** after 240 min was the highest determined integral. Thus the sum of this and the **P5** integral after 5% TFA-treatment were assumed to be 100%, since the most peptide was gained here. All other integrals were divided by this value to have the relative amount of liberated peptide **P5** in percent. See relative amounts during reduction and after DTT wash-out in 5% aq. TFA in **Figure S 13**.

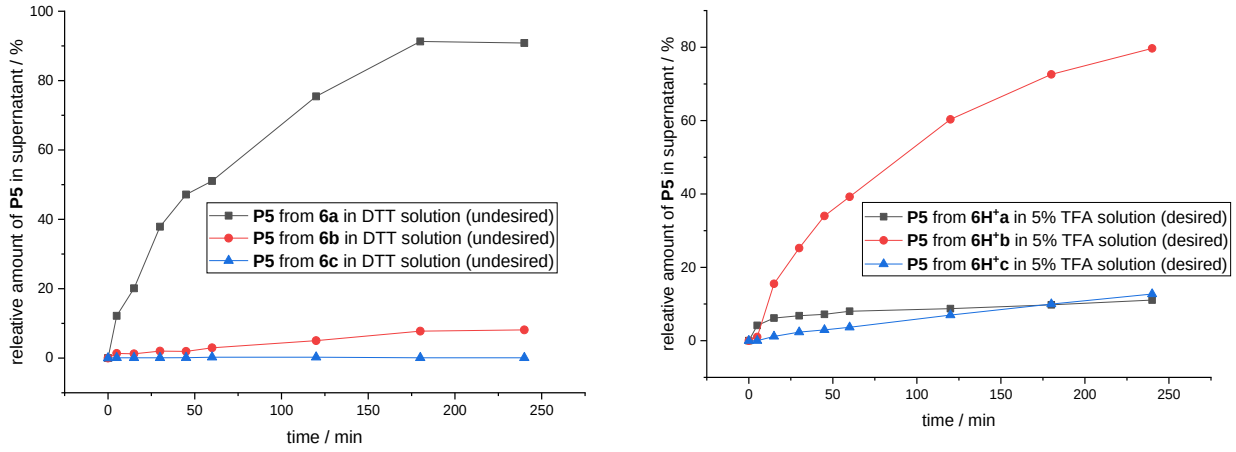


Figure S 13: Amount of released peptide **P5** in DTT supernatant (undesired, left) and after DTT wash-out in 5% aq. TFA supernatant (desired, right). Relative amounts calculated by the UV(210 nm) integral of product peak relative to the sum of DTT and TFA released peptide from **6a**.

S5 Optimizing TFA-cleavage cocktail and building blocks to mitigate side products

The following peptides were used in this study as given below in **Table S 7**. Peptide **SP6** and **SP6(S*t*Bu)** were synthesized as described for **P5** in S2.4 on page 17. In case of **SP6** Fmoc-Cys(Trt)-OH and in case of **SP6(S*t*Bu)** Fmoc-Cys(S*t*Bu)-OH was used for SPPS. To **SP6** and **SP6(S*t*Bu)** on Rink-amide PS resin was coupled **2a** as described in S3.2.2 on page 20 in 100 µmol scale. Thereafter different conditions for TFA cleavage were performed as described below. The linker tagged peptides **3a-SP6** and **3a-SP6(S*t*Bu)** were lyophilized.

Table S 10. Number, sequence, origin and molecular weight (Mw) of peptides used in this study.

number	peptide	origin	M_W (Da)
SP6	H-GCREGFLRCLHRPTVCG-NH_2	research peptide	1901.93
SP6(S*t*Bu)	H-GC(S*t*Bu)REGFLRC(S*t*Bu)LHRPTVC(S*t*Bu)G-NH_2	research peptide	2166.44

All TFA-cleavage cocktails that were evaluated in this study are listed below in **Table S 11**. Some cocktails were previously reported in literature such as Reagent K[5] or known TFA-cocktails such as Reagent L[1] and Reagent B[1]. Reagent Bel and Reagent PG was termed by us, since they gave the best results in this study.

Table S 11: Investigated TFA-cleavage cocktails; *a version of the original cocktail with less amount and ** with higher amount of scavengers.

name	vol.% TFA	vol.% TIS	vol.% H_2O	w.% PhOH	vol.% PhSMe	vol.% EDT	w.% DTT
Cocktail 1	95	2.5	2.5				
Cocktail 2	95	1	2			2	
Cocktail 3	88	2	5			5	
Cocktail 3*	84	2	8			6	
Cocktail 3**	91	2	4			3	
Cocktail 4	91	2	4				3
Reagent K	82	5	5	5	5	2.5	
Reagent L	82	2	8				8
Reagent B	88	2	5	5			
Reagent Bel	83	2	5		5	5	
Reagent Bel*	87	2	4	4		3	
Reagent PG	88	2	4				6

S5.1 Optimising TFA-cleavage cocktail

[1] https://www.peptide.com/resources/solid-phase-peptide-synthesis/cleavage-cocktails/, 13.10.2020

Having successfully erased possible sources of impurities induced by the cleavage chemistry itself, we aimed to further improve the final purity level by having a deeper look into the remaining impurities after PEC purification. We found no truncations or other impurities in the final product; the majority of remaining impurities after the PEC process originated from alkylation and oxidation reactions during TFA global deprotection. In this step a broad number of electrophiles are generated while releasing peptidic nucleophiles. We investigated several reported cleavage cocktails and made optimisations to maximise crude peptide purity (all cocktails are listed in **Table S 11**).

The peptide sequence H-AKADEVSLHKWYG-NH_2 P5 was synthesized in a 100 µmol scale on a Rink-Amide polystyrene resin. The first-generation Rink-Amide linker was chosen because it shows unwanted acidic degradation towards the 4-hydroxybenzyl cation that is readily caught by nucleophilic residues if appropriate scavengers are not present in sufficient quantities.[6] To quantify the efficiency of side-product suppression the following TFA-cleavage cocktails were investigated Cocktail 1 , Cocktail 2, Cocktail 3, Reagent K, Reagent L and Reagent B (**Table S 11**). The resin was dried after automated synthesis, and six aliquots of 10 mg of resin were treated with above mentioned TFA-cocktails in separated reactors for 1.5 hours while shaking. After cleavage, the TFA supernatant was collected and a 10-fold excess of Et_2O was added. The precipitated peptide was gained by centrifugation and the obtained pellet was washed twice with Et_2O to remove residual scavengers. The peptide pellet was dissolved in H_2O/MeCN 7:3 and lyophilized. Thereafter samples of the lyophilized product were dissolved and analysed by UPLC-MS. The corresponding peaks in the resulting UV chromatograms were integrated and identified by ESI-MS analysis. The results are shown below in **Figure S 14 A**. The results show that only the usage of Reagent K sufficiently prevents the addition of 4-hydroxyl-benzyl (+106 Da) to peptide, what may be reasoned by the thioanisol scavenging it. The amount of TIS, H_2O, EDT is important to prevent *t*Bu addition (+56 Da) in comparison of cocktail 2 and 3. EDT scavenges *t*Bu cations better then phenol when cocktail 3 is compared to Reagent B. Furthermore, comparing these two cocktails, phenol catches 4-hydroxy-benzyl cations less efficient than EDT.

In a second study two encountered side products after insufficient TFA-cleavage were in focus. First, the *t*Bu adducts on the thiol containing side chain of cysteines. The adducts result from the attack of the sulfur atom on *t*Bu electrophiles generated during TFA-cleavage. Second, the difficult removal of Pbf-protecting group from Arg residues. For this study 4x 90 mg of the peptide sequence H-GCREGFLRCLHRPTVCG-NH_2 **SP6** on Rink Amide RAM resin (assumed 20 µmol per aliquot) were added to 4 different cartridges. Each 1 mL of 4 different cleavage

cocktails were added and the cartridges were shaken rapidly. Each 30 min the shaker was switched off for 1 min and 200 µL of the cleavage cocktail was taken, the TFA-aliquots with peptide were precipitated in each 2 mL Et_2O, centrifuged and the supernatant was removed. All precipitates were dissolved in 0.8 mL H_2O/MeCN (1:1) + 0.1% TFA and analysed via UPLC-MS. The peaks in the resulting UV chromatograms were integrated, and corresponding species identified by ESI-MS analysis giving the result showing below in **Figure S 14 B**. Cocktail 3 from the first experiment described above was used again twice with a) an increased amount of water and EDT (Cocktail 3*) and b) a reduced amount of water and EDT (Cocktail 3**). A new cocktail was composed that is designed to be an improved version of Reagent K, without phenol and with TIS. Phenol catalyzes the removal of PMC-protection on arginine, but it is a toxic substance that showed no beneficial scavenging abilities in our tests and since PMC is currently not used anymore we removed it in Reagent Bel. Furthermore the amount of EDT was doubled and 2 vol% TIS was added. Trityl-cations are caught more efficiently by hydrides than by thiols.[7] Additionally, a version of Reagent Bel with a reduced amount of water, thioanisol and EDT was used (Reagent Bel**). Comparing all cocktails, the removal of Pbf is enhanced by higher amounts of TFA. Also, thioanisol catalyzes Pbf removal, as described by Guy *et al*.[8] Nevertheless, the removal of Pbf takes some time, during that time it is observed, that the intensity of peaks with +56 Da products increase. The lowest amount of *t*Bu adduct (10%), while Pbf was fully removed, was observed with reagent Bel after 90 min (**Figure S 14**). It can be concluded, that it is impossible to get no TFA-cleavage related peptidic impurities if Arg(Pbf) and Cys(Trt) residues are present in the same peptide even with an optimized cleavage cocktail, when a complete Pbf deprotection is to be achieved. This effect arises from the opposing nature of Pbf deprotection, which needs longer times, even if high TFA content and/or thioanisol is present in the cleavage cocktail, and the alkylation of already deprotected Cysteine, since the trityl group is detached very fast and the liberated thiol group is likely to attack cations during long cleavage durations. In conclusion, we found that Reagent Bel gave the best results regarding completeness of sidechain deprotection and minimal alkylation (**Figure S 14**).

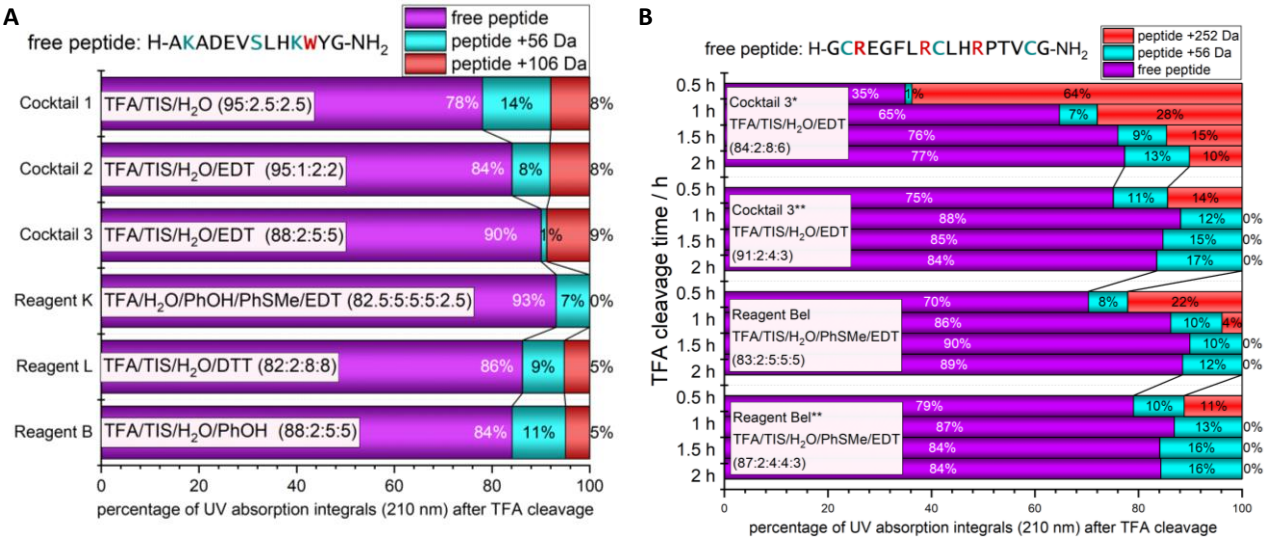


Figure S 14: A) TFA-cocktail influence in peptide product distribution, **B)** Influence of TFA additives in peptide product distribution. *higher or **lower amount of scavengers compared to original cocktail.

S5.2 Alternative SPPS building blocks to prevent side-reactions during TFA-cleavage

Independent of the choice of cleavage cocktails we often found impurities after PEC purification showing a mass of the desired product +56 Da. We found that these impurities correlated with the number of lysin and cystein residues in the sequence.

S5.2.1 Preventing tBu-Lys impurities with Fmoc-Lys(Trt)-OH

Pawlas *et al*. reported an intramolecular *N*-Boc -> *N*-*t*Bu migration during TFA-cleavage.[9] We confirmed these findings indirectly. When **P5** was synthesized using Fmoc-Lys(Boc)-OH building block during SPPS followed by PEC purification using **2b** a +56 Da side product remained as impurity in the purified product. Such side product was not observed when Fmoc-Lys(Trt)-OH was used during SPPS (**Figure S 15**).

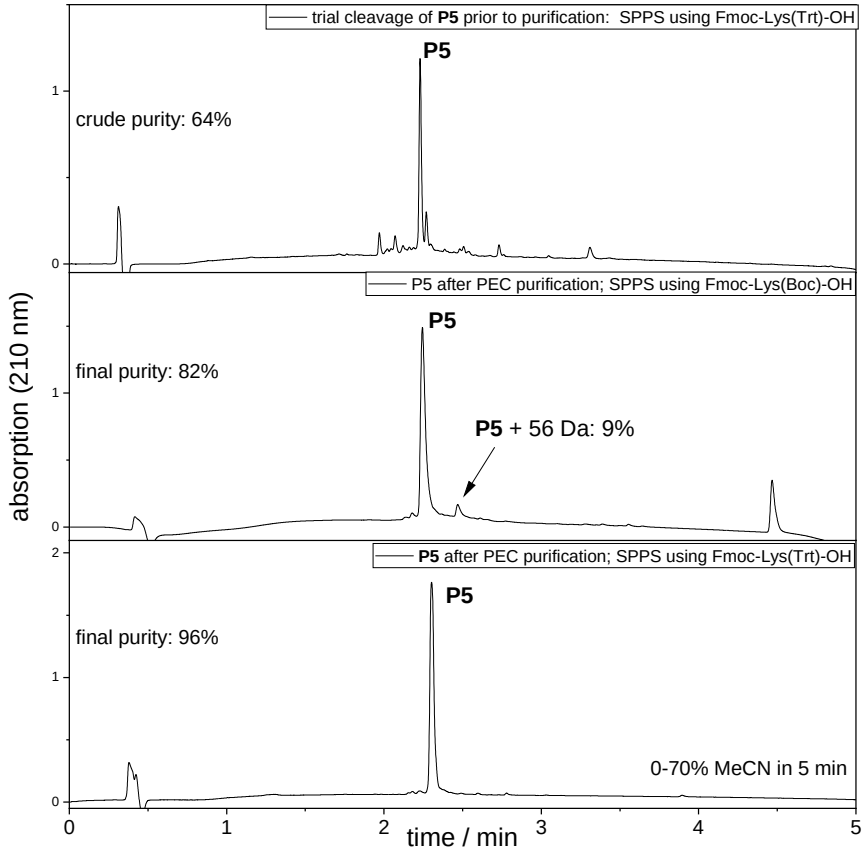


Figure S 15: Comparison of PEC purified peptide **P5** using peptide synthesized with Fmoc-Lys(Boc)-OH versus Fmoc-Lys(Trt)-OH building block.

S5.2.2 Preventing alkylation on Cys-residue with Fmoc-Cys(StBu)-OH

Disappointingly, independent of the cleavage cocktail, cysteine containing peptides always showed high amounts of alkylation side reactions, typically *t*Bu-addition as explained in S5.1 staring from page 49. We therefore investigated Fmoc-Cys(S*t*Bu)-OH as building block to synthesize Cys-containing peptides. The S*t*Bu-protection on Cys was selected to prevent alkylation on Cys during TFA-cleavage, because the disulfide remains intact during the process. Additionally, the S*t*Bu protection can be conveniently removed in the PEC-process when reducing the azide with DTT. As a proof-of-principle the peptide **SP6** (H-GCREGFLRCLHRPTVCG-NH_2) was synthesized using either Fmoc-Cys(Trt)-OH or Fmoc-Cys(S*t*Bu)-OH as the Cys building block resulting in crude **SP6** or **SP6(S*t*Bu)**.

In accordance with TFA-cocktail study above, we tested the cocktails given above with an odourless version of Reagent Bel, Reagent PG TFA/H_2O/DTT/TIS (88:4:6:2) with peptide **SP6** and **SP6(S*t*Bu)** both having 3x Arg(Pbf).

Investigation of the scavengers' effects. 8 x 10 µmol (45 mg resin) of the Cys(Trt) and 8 x 10 µmol (45 mg resin) of the Cys(StBu) containing linker-modified peptide on resin were added to fritted-cartridges. 1 mL of each

cleavage cocktail was poured into two cartridges of each peptide to separate between one and two hours of shaking time. After ether precipitation, centrifugation, and removal of the ether supernatant for all peptides, precipitates were dissolved, purified by PEC and the product was analysed by UPLC-MS.

Both peptides were purified using linker **2b** as described in S3 on page 19 gaining the desired peptide from the Cys(Trt) SPPS in a purity of 83% where *t*Bu-adducts and oxidation were remaining impurities, while the peptide from the Cys(S*t*Bu) SPPS was obtained with a much higher purity of 97% (**Figure S 16**).

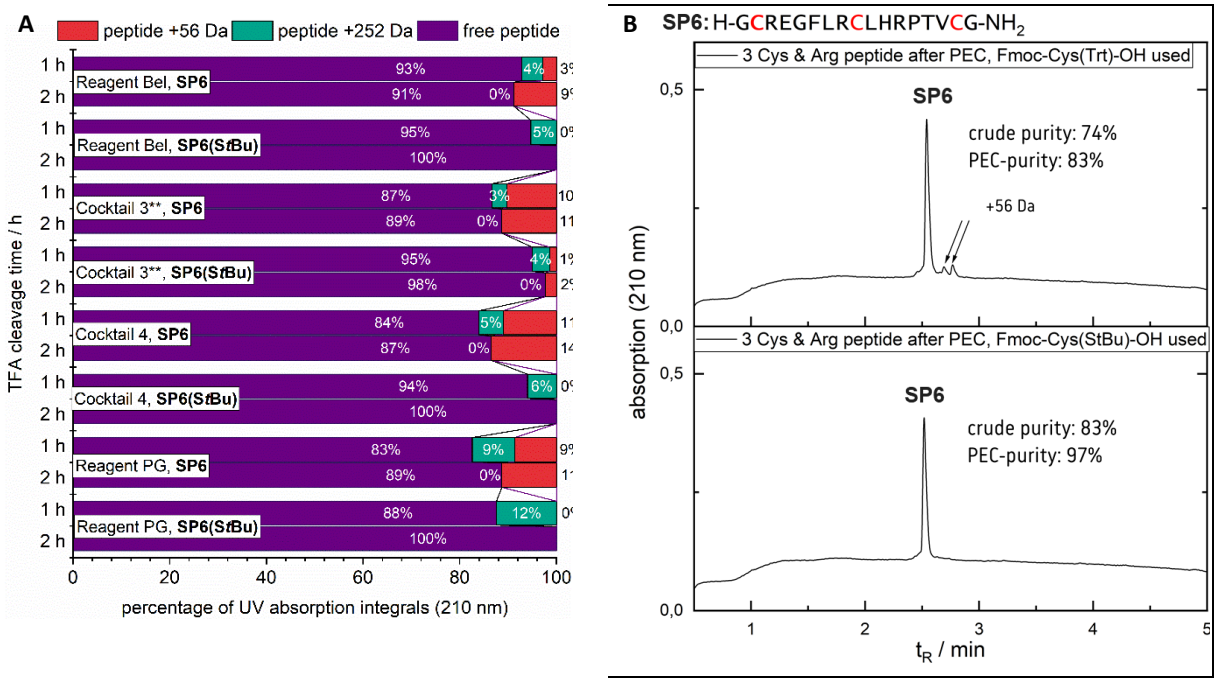


Figure S 16, A: Influence of TFA-cleavage cocktails and time on crude peptide purity using peptide **SP6** or **SP6(StBu)**. **B**: Comparison of PEC purified peptide **SP6** using peptide synthesized with Fmoc-Cys(Trt)-OH **SP6** versus Fmoc-Cys(Trt)-OH building block **SP6(StBu)**.

S6 Purification of a personalised peptide vaccine set

50 µmol crude peptide on SPPS resin of each of the 20 peptides (see Table 3 Main Text or **Table S 1**on page 17), synthesized as described above in section S2.4, was used for the parallel PECpurification of this set. In the following, linker-coupling and TFA-cleavage is described for the 50 µmol, while the purification was carried out for only 1x 25 µmol peptide, as described.

Linker coupling and TFA cleavage (50 µmol scale): Linker **2b** was attached to the N-terminus of the peptide as the last building block. For this, the peptide on resin in the SPPS cartridge was treated with DMF for 15 min. 150.5 mg (200 µmol, 4 eq) linker **2b** and 43 mg (300 µmol, 6 eq) Oxyma was dissolved in 666 µL DMF. 52.5 µL (300 µmol, 6 eq) DIPEA was added and the solution was transferred into the cartridge. The mixture was shaken at rt for 2 h. The solution was removed by vacuum suction and the beads were washed 3x with 1 mL DMF and 3x with 1 mL DCM. TFA cleavage was performed for 2 h using 5 mL TFA/H_2O/DTT/TIS (84:8:6:2). The solution was eluted into a 50 mL centrifuge tube with 45 mL chilled diethyl ether to precipitate the crude linker-coupled peptide. Centrifugation and one ether wash yielded the crude material. Methionine-containing peptides **P8**, **P10**, **P12-P15**, **P18**, **P21**, **P23** and **P25** were redissolved in 5 mL Reagent Bel and 70 µL of TMSBr was added and the centrifuge tube was shaken for 15 min. Thereafter 45 mL chilled diethyl ether was added to precipitate the crude linker-coupled peptide. Centrifugation and one ether wash yielded the crude material. All peptide pellets were dried in vacuo. Each sample of crude material was split into two equal parts (2x 25 µmol) and 1x 25 µmol of each peptide was purified as described in the following paragraph.

PEC purification: The crude linker-coupled peptides were dissolved in 1.125 mL DMSO and 125 µL IB(GdmCl)was added. 0.75 mL modified agarose beads suspension was transferred into 5 mL fritted cartridges and the supernatant was removed. The beads were washed 3x with 2 mL water, 3x with IB and 1x with DMSO. 375 µL DMSO was added to each cartridge, followed by the peptide solution. The mixture was shaken at rt for 90 min. The supernatant was removed by filtration, the beads were washed 3x with 2 mL DMSO and then incubated with 1.25 mL 2 w% L-cysteine in IB for 15 min. The solution was removed, and the beads were washed 3x with 2 mL 0.9 M GdmCl in DMSO and 3x with 0.1 M aq. NaCl/EtOH (3:7). Peptides without Cys residues were incubated with 2.5 mL 1:1 MeCN/50 mg DTT dissolved in 5 w% aq. $NaHCO_3$ (0.6 M, pH 8) for 15 min, while the Cys(StBu) containing peptides **P12** and **P17** were incubated for 60 min to also remove the StBu-protecting group quantitively. The solution was removed, and the beads was washed 3x with water and 3x with MeCN. Thereafter,

0.5 mL TFA/H_2O (4:6) was added to each cartridge to initiate linker cleavage. After incubation for 60 min, 0.5 mL TFA was added and the solution was eluted into a 15 mL centrifuge tube. The beads were further eluted with 2x 0.5 mL TFA/H_2O 95:5 and the solutions collected in the same tube. The purified peptide was precipitated upon addition of chilled diethyl ether. The sample was centrifuged, and the ether phase discarded. The air-dried precipitates were dissolved in MeCN/H_2O (3:7) and lyophilized to give the purified peptide as a powder.

All peptides were gained as white fluffy powder. The weight of the peptides, the recovery of the PEC purification (calculated with equation S1, page 24) the calculated and found ESI-MS signals are listed below in **Table S 12**.

Table S 12: The measured weight, recovery (equation S1, page 24), calculated and found ESI-MS signals of the 20 peptides of the personalized vaccine set.

No.	Sequence	MW	product weight / recovery	calc. ESI-MS signals	found [m/z]
P6	H-GWVKPIIIGHHAYGDQYRAT-NH_2	2280.19	19.2 mg / 45%	MH^{2+}: 1141.10, MH^{3+}: 761.06	1141.54, 761.56
P7	H-TLYEQEIEV-NH_2	1121.56	14.6 mg / 56%	MH^{+}: 1122.56, MH^{2+}: 561.78	1122.61, 561.83
P8	H-HGSRKNITDMVEGAKKANG-NH_2	2011.04	16.3 mg / 52%	MH^{2+}: 1006.89, MH^{3+}: 671.35	1006.89, 671.80
P9	H-SLLNQPKAV-NH_2	967.58	24.2 mg / 100%	MH^{+}: 968.58, MH^{+}: 484.79	968.72, 485.04
P10	H-EDPYLFELPVLKYLDMGTT-NH_2	2242.12	35.6 mg / 73%	MH^{2+}: 1122.06, MH^{+}: 748.37	1122.58, 748.73
P11	H-ALAVLSNYDA-NH_2	1034.54	22.1 mg / 93%	MH^{+}: 1035.54, MH^{2+}: 518.27	1035.63, 518.44
P12	H-TMEDKIYDQQVTKQCLCF-NH_2	2191.01	7.0 mg / 69%	MH^{2+}: 1096.51, MH^{3+}: 731.34	1095.88, 731.14
P13	H-YSYPETPLYMQTASTSYYE-NH_2	2291.99	22.9 mg / 69%	MH^{2+}: 1147.00, MH^{3+}: 765.00	1147.52, 765.29
P14	H-KVGYTERQRWDFLSEASIM-NH_2	2314.15	3.4 mg / 81%	MH^{2+}: 1158.08, MH^{3+}: 772.38	1158.53, 772.80
P15	H-RLRMREHMMKNVDTNQD-NH_2	2172.05	19.1 mg / 76%	MH^{2+}: 1087.03, MH^{3+}: 725.02	1087.51, 725.49
P16	H-VYEKNGYIYF-NH_2	1293.64	16.8 mg / 90%	MH^{2+}: 647.82	648.48
P17	H-ALAVLCNYDA-NH_2	1050.52	20.2 mg / 81%	MH^{2+}: 1051.52, MH^{3+}: 526.26	1051.57, 526.38
P18	H-ALVPPSKRKMWVVSPAEKA-NH_2	2092.20	27.0 mg / 72%	MH^{2+}: 1047.10, MH^{3+}: 698.40	1047.60, 698.90
P19	H-ISTPTPTIVHPGSLPLHLG-NH_2	1935.09	21.8 mg / 60%	MH^{2+}: 968.55, MH^{3+}: 646.06	968.97, 646.53
P20	H-IVQENNTPGTYLLSVSARD-NH_2	2075.06	24.2 mg / 69%	MH^{2+}: 1038.53, MH^{3+}: 692.69	1038.96, 693.12
P21	H-RFHMKVSVYLLAPLREALS-NH_2	2228.26	18.0 mg / 57%	MH^{2+}: 1115.13, MH^{3+}: 743.75	1115.66, 744.26
P22	H-ENLKQNDISAEFTYQTKDA-NH_2	2213.06	26.2 mg / 82%	MH^{2+}: 1107.53, MH^{3+}: 738.69	1108.00, 739.12
P23	H-YMMPVNSEV-NH_2	1067.48	15.7 mg /100%	MH^{+}: 1068.48, MH^{2+}: 534.74	1068.56, 534.88
P24	H-TNDVKTLADLNGVIEEEFT-NH_2	2106.05	21.0 mg / 63%	MH^{2+}: 1054.03, MH^{3+}: 703.02	1054.50, 703.45
P25	H-SAWLFRMWYIFDHNYLKPL-NH_2	2498.27	19.3 mg /49%	MH^{2+}: 834.09, MH^{3+}: 625.57	834.28, 625.90

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S7 DFT-based calculations

Density functional theory (DFT) was used to calculate the pKa values of relevant heteroatoms of azido-linker propylamine conjugates 9a, 9b and 9c and their reduced aniline forms **12a**, **12b**, **12c**. Thereafter partial charges of all relevant atoms in the benzylic carbamate system were calculated.

S7.1 Calculation of proton affinities of linker heteroatoms

Non-protonated linker propylamine conjugates (**9**, **12**) were constructed and structure optimization was performed using DSD-PBE-NL/TZVP//PBE0-D3BJ/SVP basis set. After a minimum energy structure was found, the desired protonated molecules were generated by bringing protons into proximity of the heteroatoms of interest (Figure S 17).

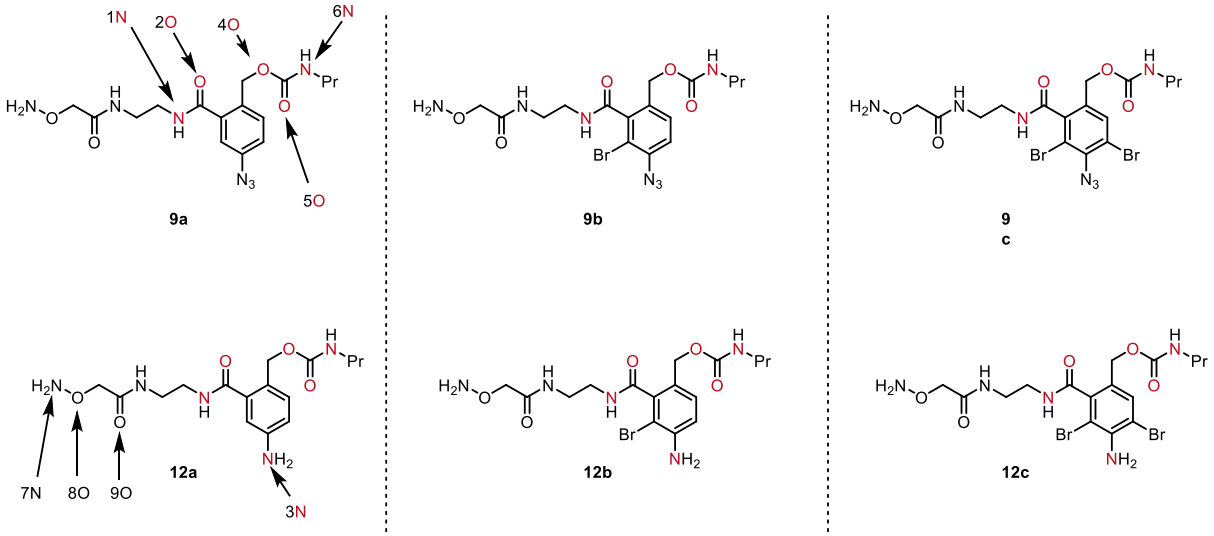


Figure S 17: Linker propylamine conjugate with distinct investigated protonation sites, marked with red.

The resulting 15 structures for **9** and 18 structures for **12** were optimized again. Stabilization by hydrogen bonding could be observed for all protonation sites. Of note, protonation on 4O resulted in all cases in protonation of 2O, where the hydrogen shows bonding towards 4O. All optimized structures except the none-protonated version of **9** are illustrated in **Table S 13** for **9a**/**12a**, in **Table S 15**: Structures of di-brominated linker conjugate **9c** and **12c** in different protonation stats. for **9b**/**12b** and in **Table S 15** for **9c**/**12c**.

Table S 13: Structures of non-brominated linker conjugate 9a and 12a in different protonation stats.

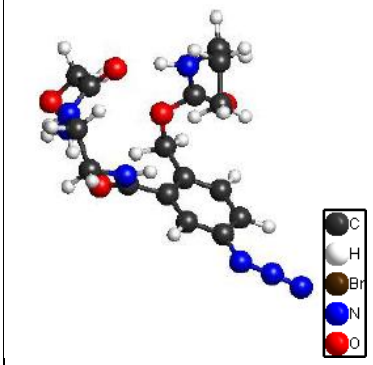
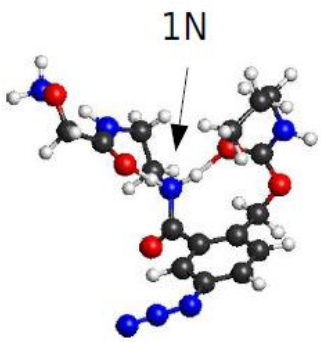
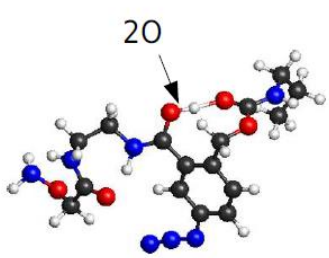
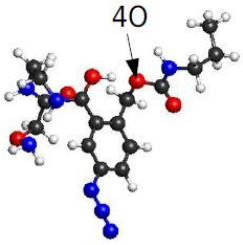
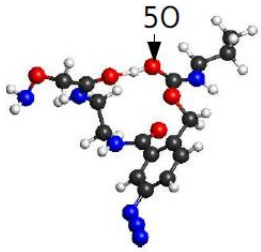
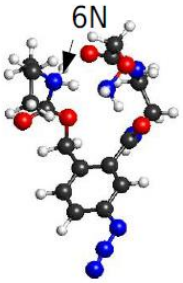
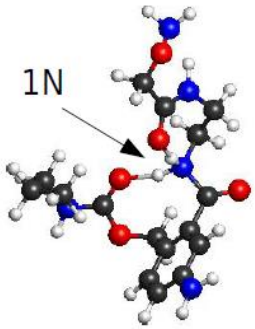
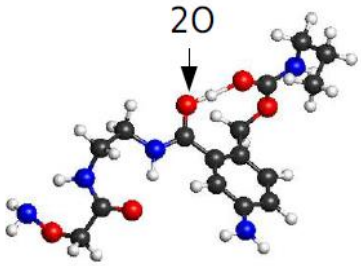
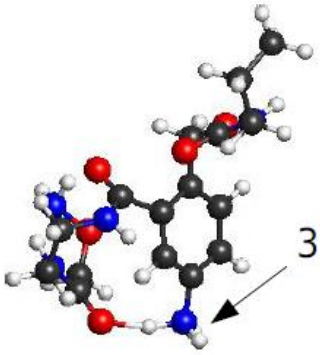
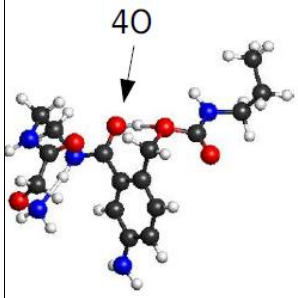
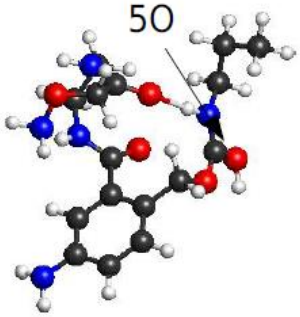
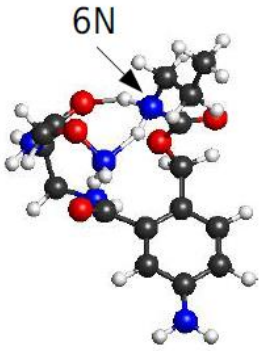
		
Non-protonated optimized structure of **9a**	Protonation of **9a** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **9a** on 2O, hydrogen bonding: 2O →5O
		
Protonation of **9a** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **9a** on 5O, hydrogen bonding: 5O →9O	Protonation of **9a** on 6N, hydrogen bonding: 6N →9O, 6N → 7N
		
Protonation of **12a** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **12a** on 2O, hydrogen bonding: 2O →5O	Protonation of **12a** on 3N, hydrogen bonding: 3N→7O
		
Protonation of **12a** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **12a** on 5O, hydrogen bonding: 6N →9O	Protonation of **12a** on 3N, hydrogen bonding: 6N→9O, 6N→7N

Table S 14: Structures of mono-brominated linker conjugate **9b** and **12b** in different protonation stats

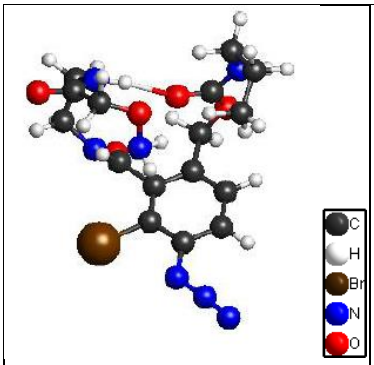
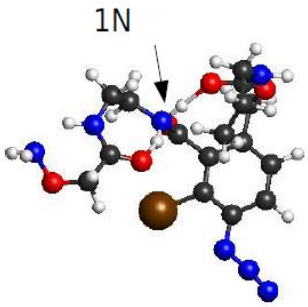
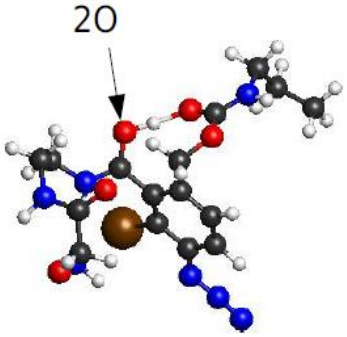
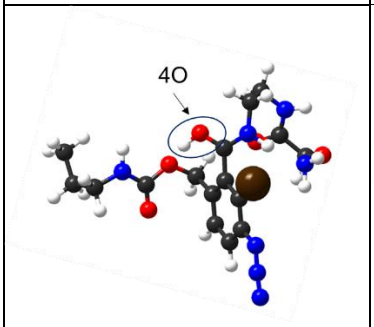
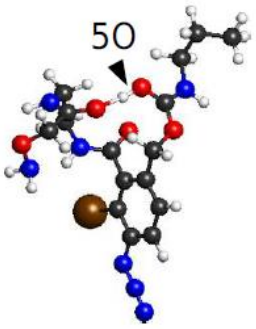
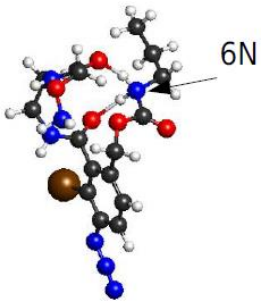
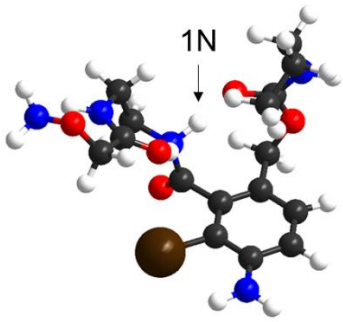
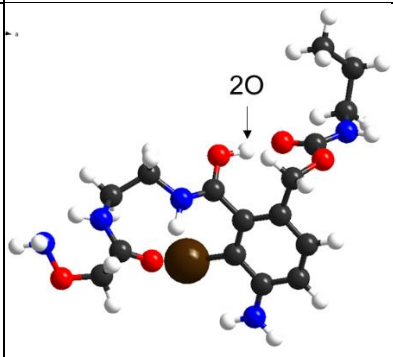
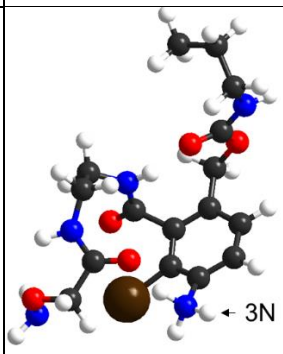
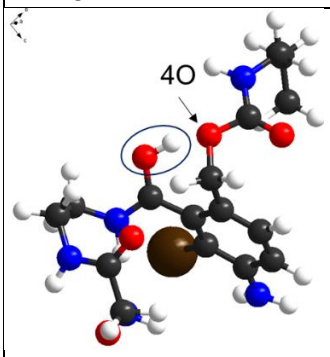
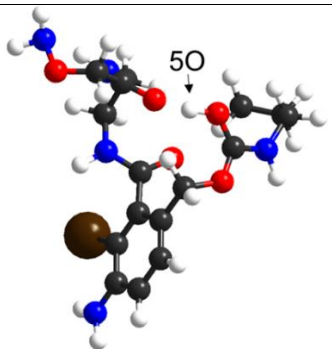
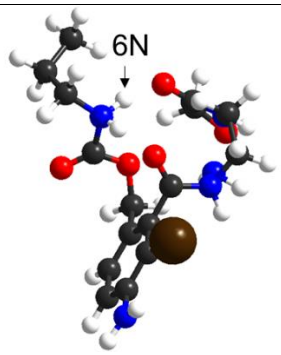
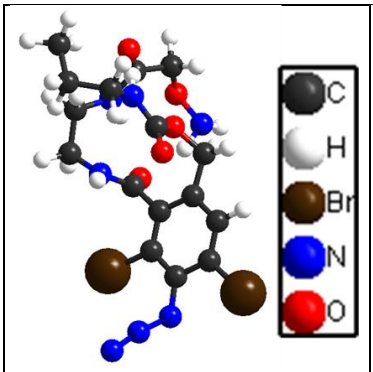
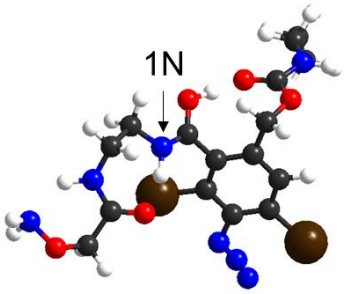
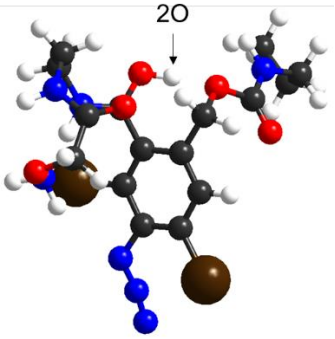
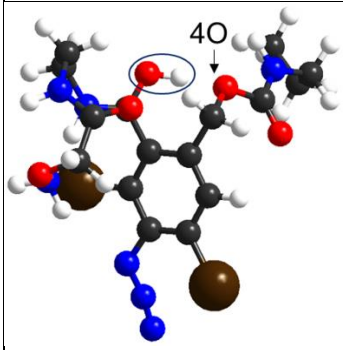
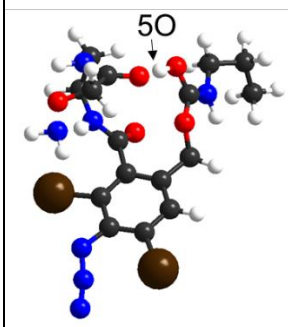
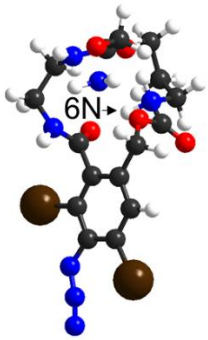
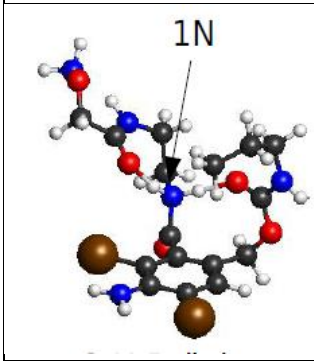
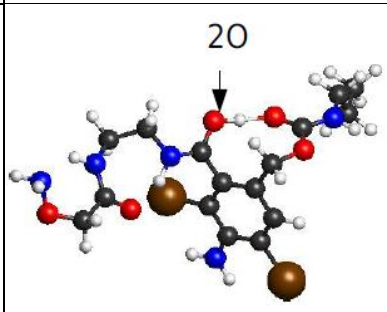
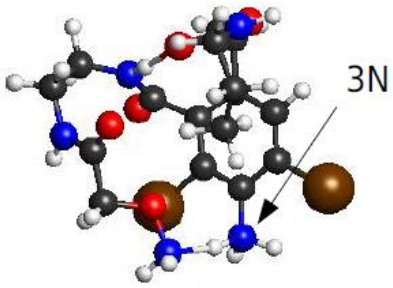
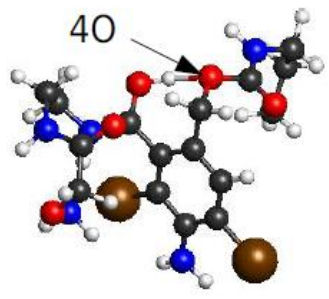
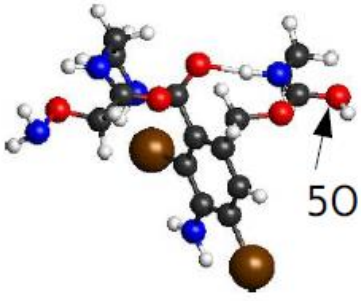
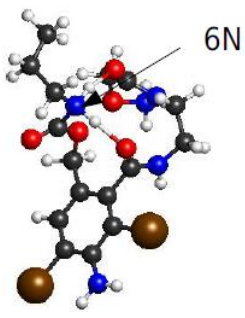
		
Non-protonated optimized structure of **9b**	Protonation of **9b** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **9b** on 2O, hydrogen bonding: 2O →5O
		
Protonation of **9b** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **9b** on 5O, hydrogen bonding: 5O →2O	Protonation of **9b** on 6N, hydrogen bonding: 6N →9O, 6N → 7N
		
Protonation of **12b** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **12b** on 2O, hydrogen bonding: 2O →5O	Protonation of **12b** on 3N, hydrogen bonding: 3N→7O
		
Protonation of **12b** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **12b** on 5O, hydrogen bonding: 5O →2O	Protonation of **12b** on 3N, hydrogen bonding: 6N→9O, 6N→7N

Table S 15: Structures of di-brominated linker conjugate **9c** and **12c** in different protonation stats.

		
Non-protonated optimized structure of **9c**	Protonation of **9c** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **9c** on 2O, hydrogen bonding: 2O →5O
		
Protonation of **9c** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **9c** on 5O, hydrogen bonding: 5O →2O	Protonation of **9c** on 6N, hydrogen bonding: 6N →9O, 6N → 7N
		
Protonation of **12c** on 1N, hydrogen bonding: 1N→5O, 1N→9O	Protonation of **12c** on 2O, hydrogen bonding: 2O →5O	Protonation of **12c** on 3N, hydrogen bonding: 3N→7O, 1N→5O
		
Protonation of **12c** on 4O resulting in 2O protonation, hydrogen bonding: 2O→4O	Protonation of **12c** on 5O, hydrogen bonding: 6N →2O	Protonation of **12c** on 3N, hydrogen bonding: 6N→9O, 6N→9O

The calculated energies of protonated species were subtracted from the energies of the none-protonated structure resulting in a calculated proton affinity (PA) for **9** (**Figure S 18**) and **12** (**Figure S 19**).

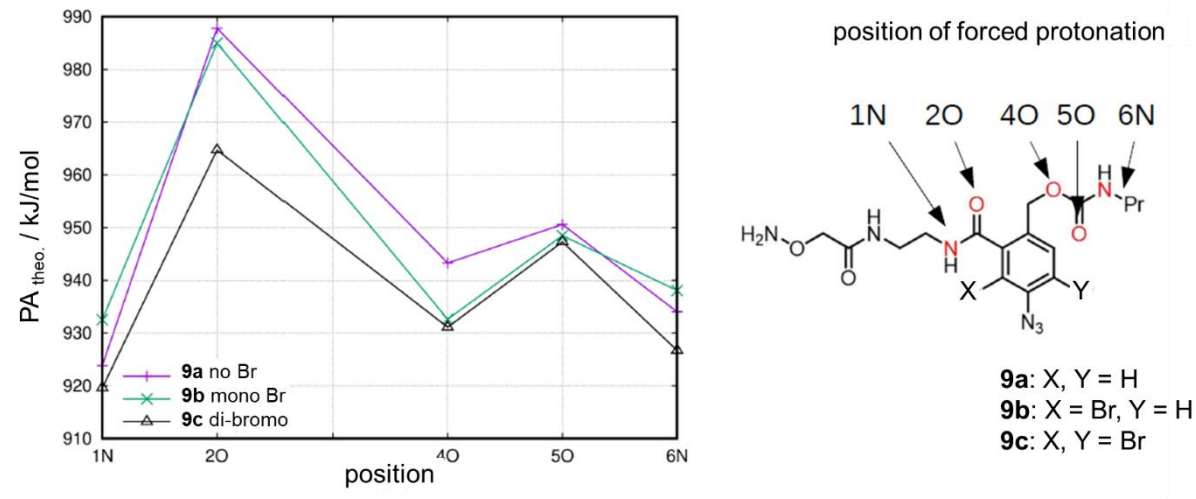


Figure S 18: Calculated proton affinities ($PA_{theo.}$) of various hetero atoms in **9a**, **9b** and **9c.**

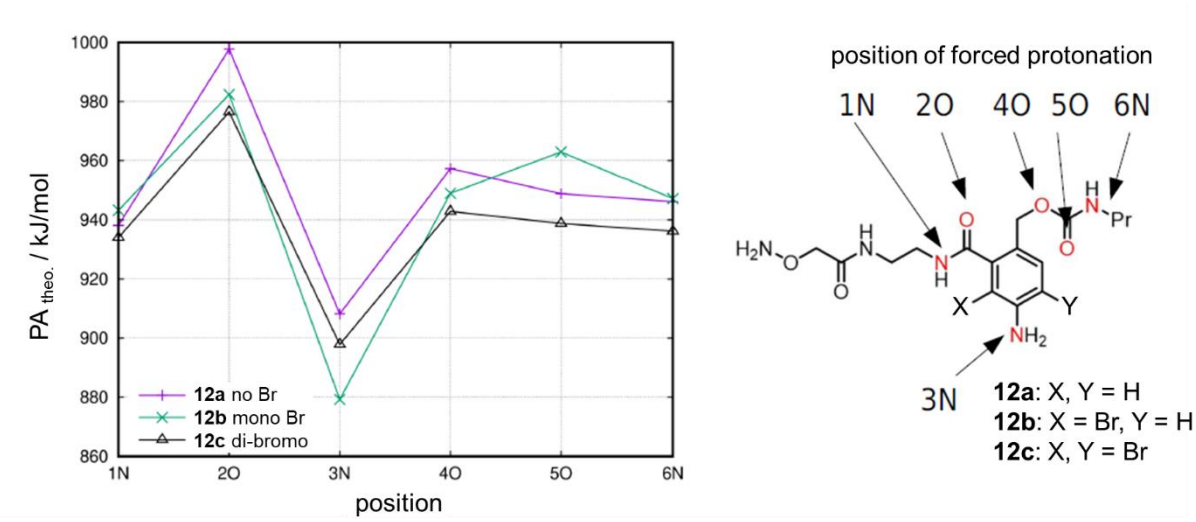


Figure S 19: Calculated proton affinities ($PA_{theo.}$) of various hetero atoms in **12a**, **12b** and **12c.**

Experimentally determined pKa values from the literature of molecules given in the table on the left side of **Figure S 20** were correlated with calculated proton affinities as calculated for **9** and **12**, giving the correlation shown in. The calculated values can be found in **Figure S 21**. Of note, aniline nitrogen had the lowest pKa and 2O and 5O have the highest pKa thus the highest proton affinities, most likely due to strong hydrogen bonding between those sites. After protonation of 2O in all **9**, **12** and 5O in **9b** and **12b** those two atoms have a close distance due to hydrogen bonding (**Figure S 21** C). Differences between brominated species were very little.

A

	pKa (exp.)	PA [kJ/mol]
Acetone + H^+	-7.50	812.00
Dimethylether + H^+	-3.60	828.40
Acetaldehyde + H^+	-8.00	768.50
Diethylester + H^+	-6.50	837.70
Acetic acid + H^+	-6.00	783.70
Ameisensäure	3.77	1449
Formic acid	4.20	1423
Acetic acid	4.76	1457
Proline	1.99	1431
H_3PO_4	2.16	1383
Oxalic Acid	1.23	1356

B

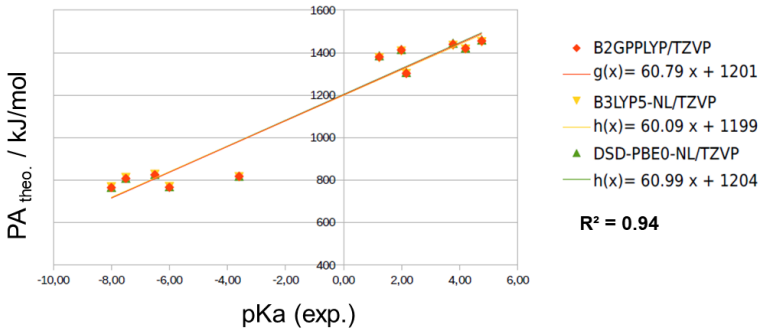
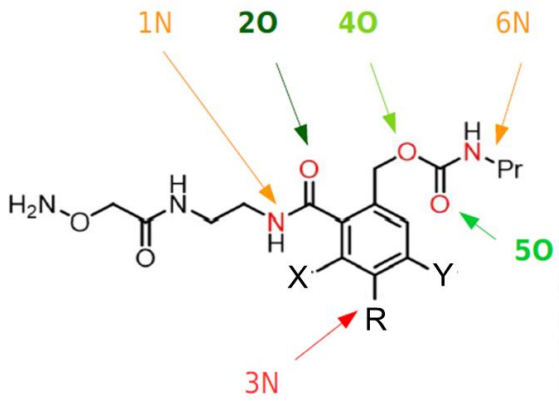


Figure S 20, **A**: Experimentally determined pKa values of molecules given in the table on the left side and calculated proton affinities as determined for **9** and **12** with the DSD-PBE-NL/TZVP basis set. **B**: Linear correlation of PAs calculated with different basis-sets, giving the correlation with corresponding linear regressions shown on the right side.

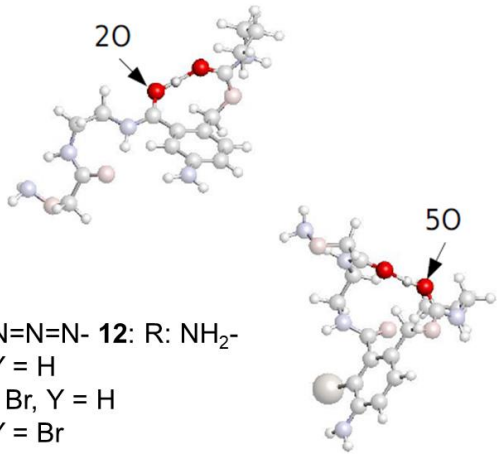
A

Pos.	no bromo pKa 9a	no bromo pKa 12a	mono bromo pKa 9b	mono bromo pKa 12b	di bromo pKa 9c	di bromo pKa 12c
1N	-4.59	-4.36	-4.45	-4.28	-4.66	-4.43
2O	-3.54	-3.38	-3.59	-3.63	-3.92	-3.73
3N		-4.85		-5.32		-5.02
4O	-4.27	-4.04	-4.45	-4.18	-4.47	-4.28
5O	-4.15	-4.18	-4.19	-3.95	-4.21	-4.35
6N	-4.43	-4.23	-4.36	-4.21	-4.55	-4.39

B



C



9: R: N=N=N- **12**: R: NH_2-
a: X, Y = H
b: X = Br, Y = H
c: X, Y = Br

Figure S 21, **A**: Calculated pKa values for different protonation sides of **9** and **12**. Red indicates high, orange medium and green low acidity resulting from low, medium and high proton affinity. **B**: Structure of **9** and **12** with colour coded heteroatoms. **C**: Strong hydrogen-bonding of 2O→5O and 5O→2O after protonation of 2O or 5O.

S7.2 Calculation of partial charges of benzylic carbamate

To gain a more detailed understanding why stability and lability in dependence of pH is so different between the non-, mono- and di-brominated linker peptide conjugates (**3a**, **3b** and **3c**; **6a**, **6b** and **6c**) additionally to the pKa calculation also partial charges of atoms in the benzylic carbamate moiety were calculated.

The above mentioned optimized structures were used and partial charges were calculated as previously described by Mulliken.[10] Most of the charges can be found in **Figure S 22**. The red colour indicates negative, white neutral and blue positive partial charge on the corresponding atoms. The sum of all carbon atoms in the phenyl ring has been calculated and used to judge electron density in the aromatic ring. It can be concluded that the electron density in the ring decreases upon bromination. Electron density is least in the 4-azido derivates (**9**). If electron density on benzylic carbon is low (around zero) no decomposition via 1,6-elimination might occur. Protonation at 2O strongly increases electron density at benzylic carbon, thus enabling benzylic scission, most likely via 1,6-elimination since the electron density of the phenyl ring is only slightly decreased.

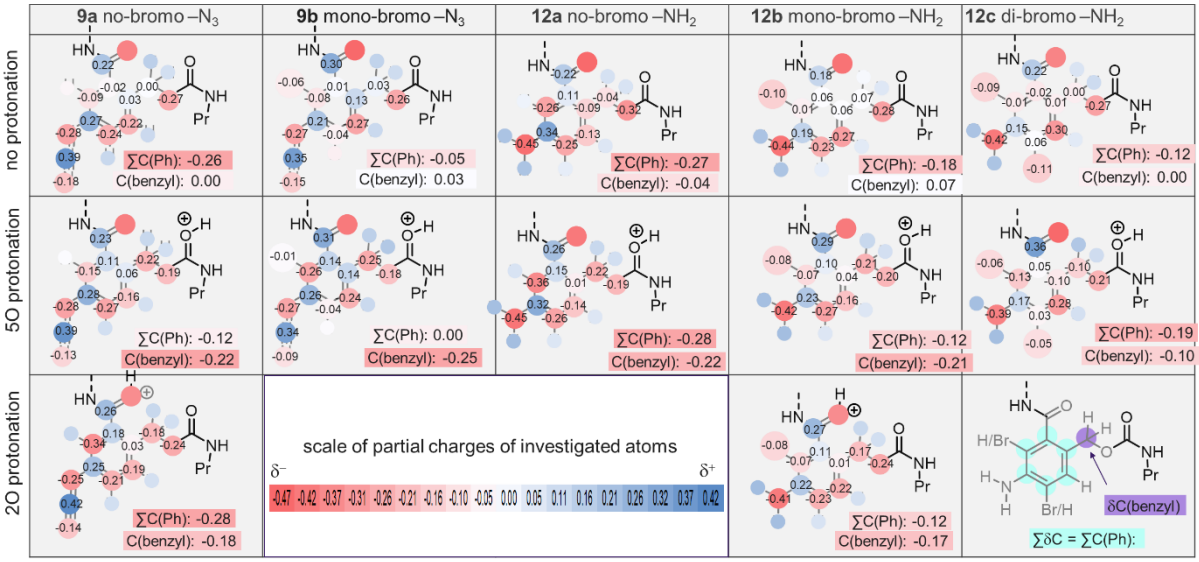


Figure S 22: Partial charges on atoms of the benzylic carbamate system calculated as described by Mulliken.[10]

S7.3 Final interpretation of Investigation of linker cleavage with DFT calculations

By using density functional theory (DFT) we wanted to get a deeper understanding of how bromination stabilizes linker conjugates during TFA cleavage and how it prevents spontaneous decomposition of 4-amidobenzyl carbamate **6b** and **6c** after reduction. During acidic detachment of **3** and after reduction of azide to **6**, brominated derivates **b** and **c** show decreased electron density of the phenyl ring and also slightly on the benzylic carbon compared to the non-brominated derivates **3a** and **6a**. Thus, in brominated derivates the electron

deficiency hampers decomposition via a Cbz-type mechanism in **3b**/**c** (Fig. 2 a) and 1,6-elimination (Fig. 3 a) at neutral to basic pH in **6b**/**c**. Surprisingly, the most basic heteroatom in all calculated linker-conjugates (**9**/**12**) is the oxygen of the *ortho*-amido group, second most basic atoms are the oxygen atoms of the carbamate group, the aniline nitrogen is least basic, by two magnitudes. After protonation at the *ortho*-amide or carbamate, hydrogen bonding of the additional proton between those two groups have always been calculated (S8.1). In summary the calculations suggest that the brominated linker conjugates **6b**/**c** are stable towards an 1,6-elimination. If proton concentration rises the *ortho*-amide oxygen is protonated first shuffling the proton to the carbonyl oxygen of the carbamate. Protonation at this site pulls electron density from the aromatic core towards the benzylic carbon enabling 1,6-elimination of the brominated linkers and thus release of the peptide.

S8 References

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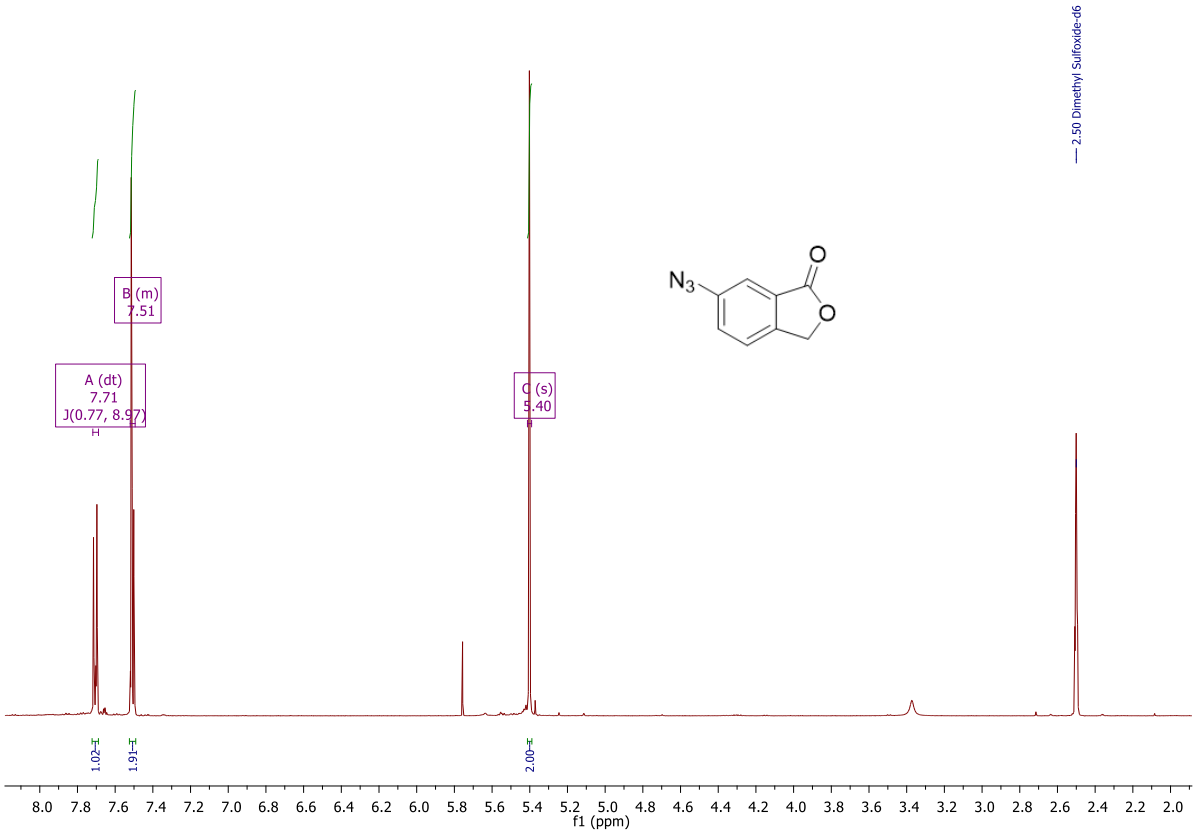
8. C. A. Guy and G. B. Fields, in *Methods in Enzymology*, Academic Press, 1997, vol. 289, pp. 67-83.
9. J. Pawlas and U. Törnvall, *Proceedings of the 33rd European Peptide Symposium*, 2014, 108-109.
10. R. S. Mulliken, *J. Chem. Phys.*, 1955, **23**, 1833-1840.

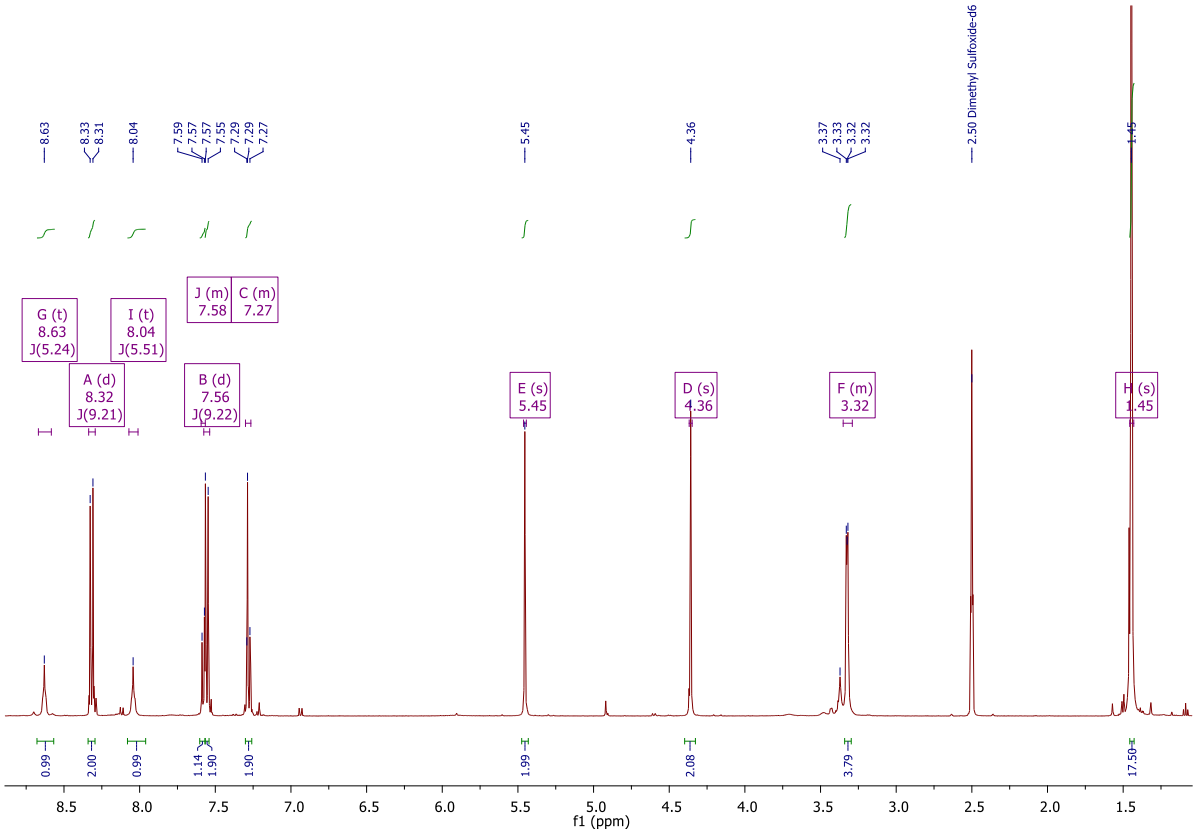
S9 Appendix

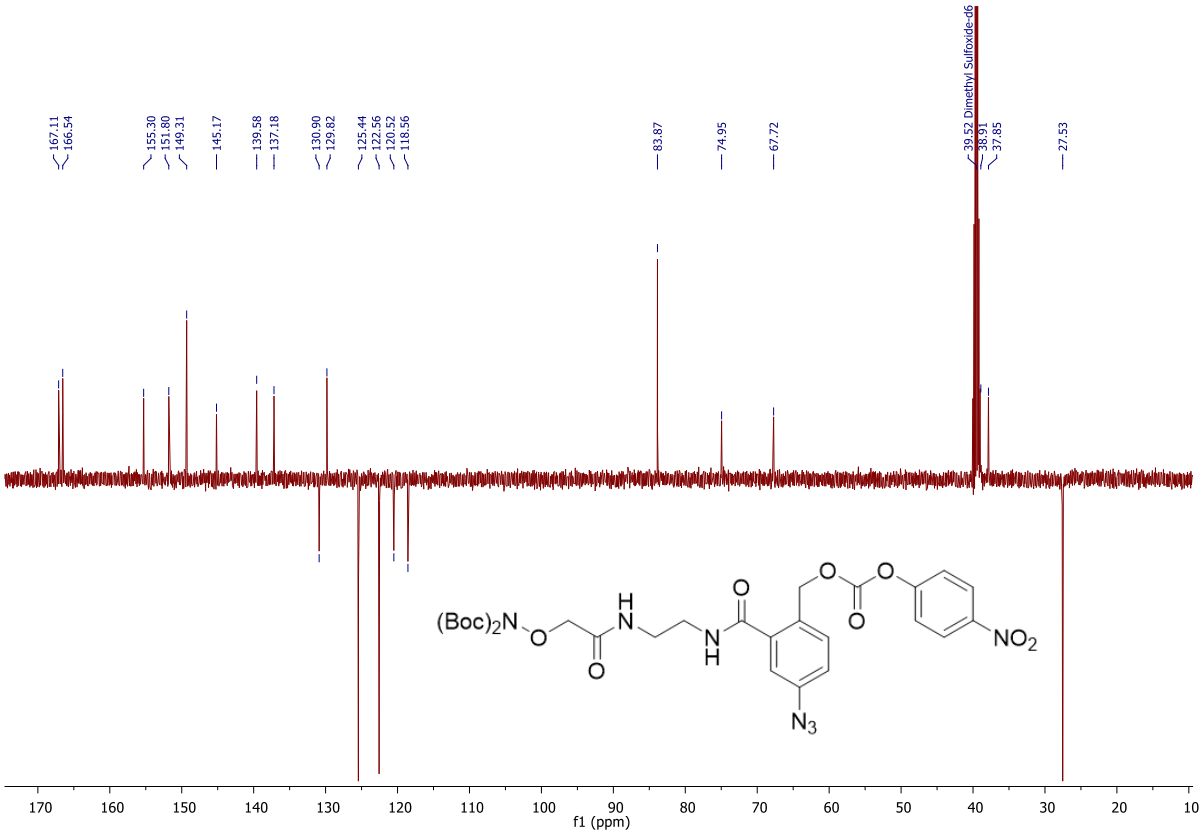
S9.1 NMR spectra

^{1}H NMR spectrum of S2a

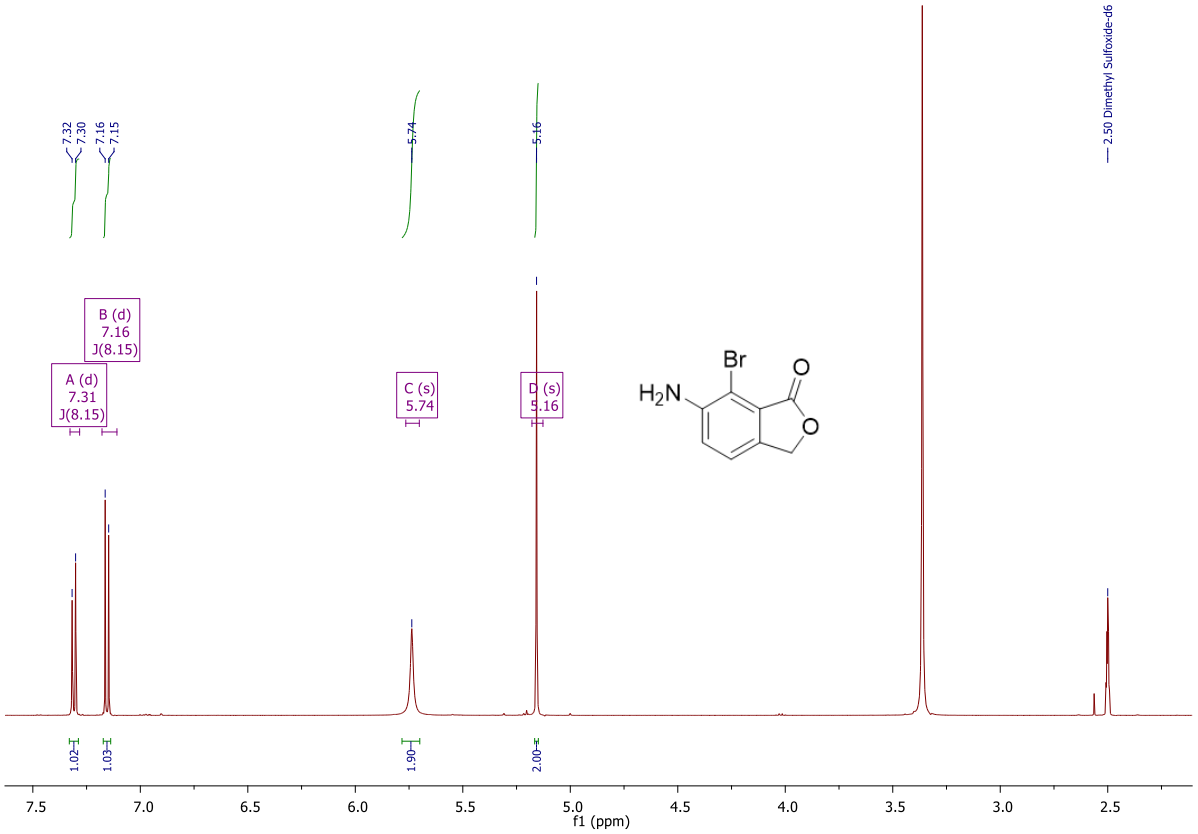


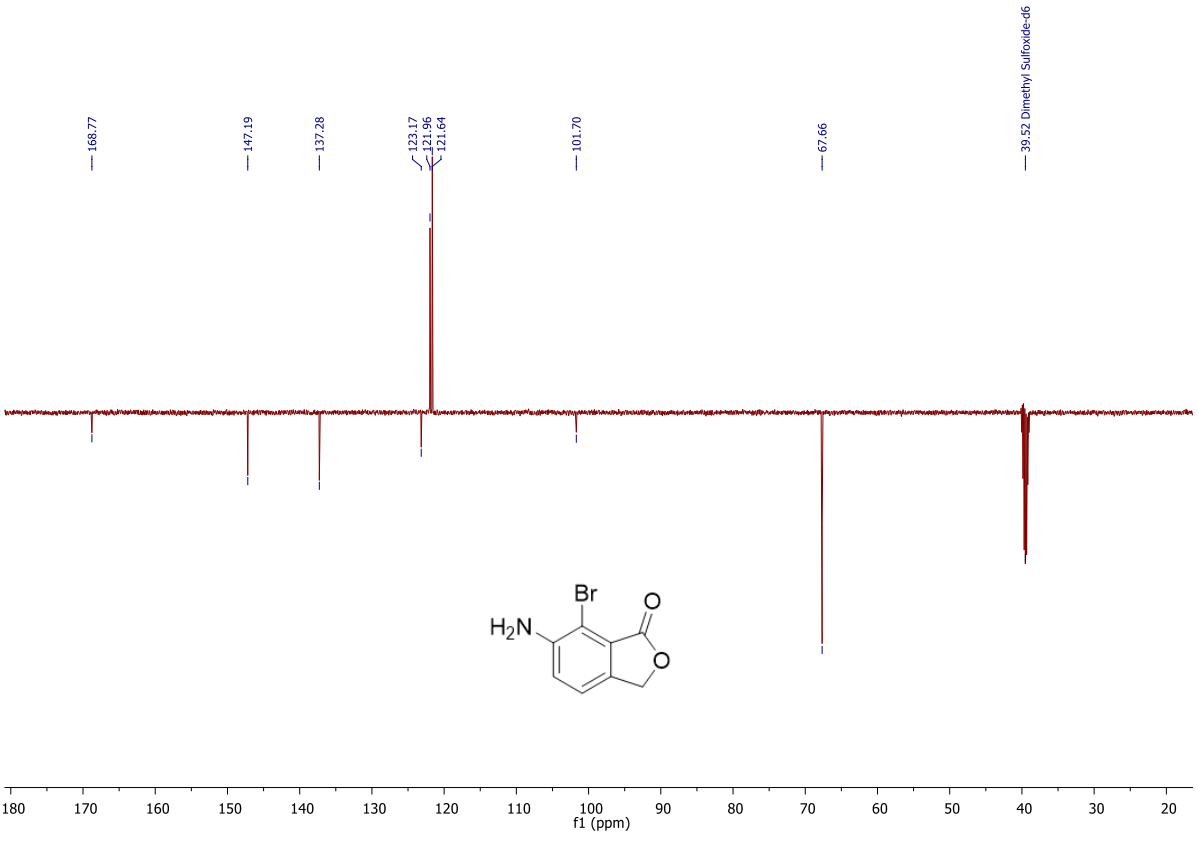
^{1}H & ^{13}C NMR spectrum of 2a



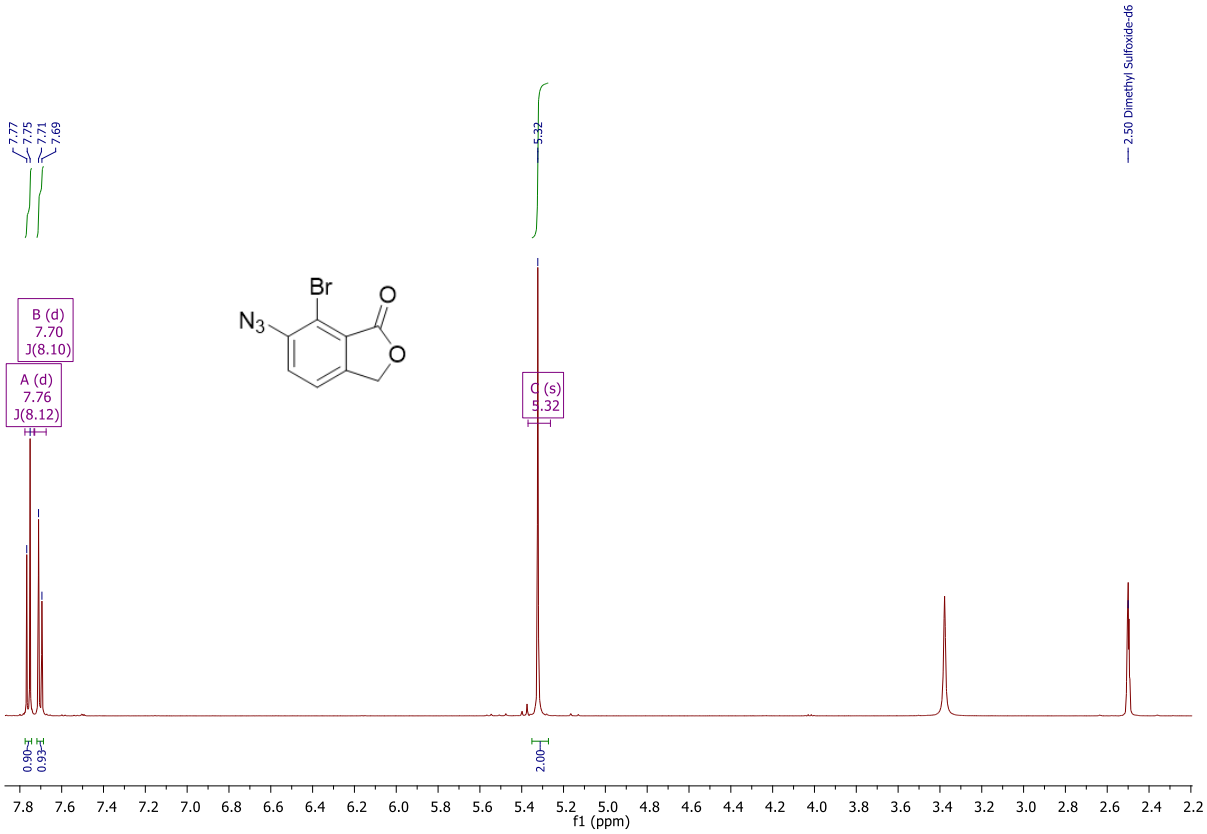


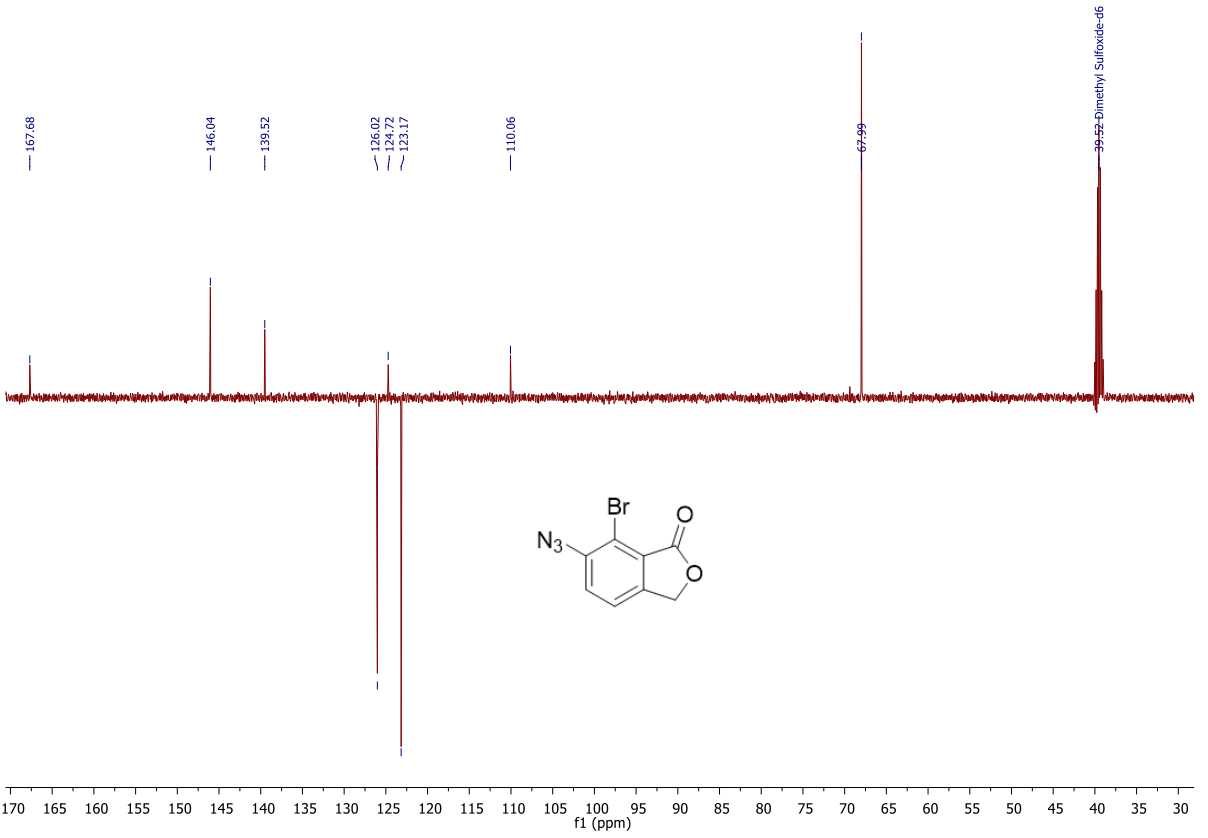
^{1}H & ^{13}C NMR spectrum of S1b



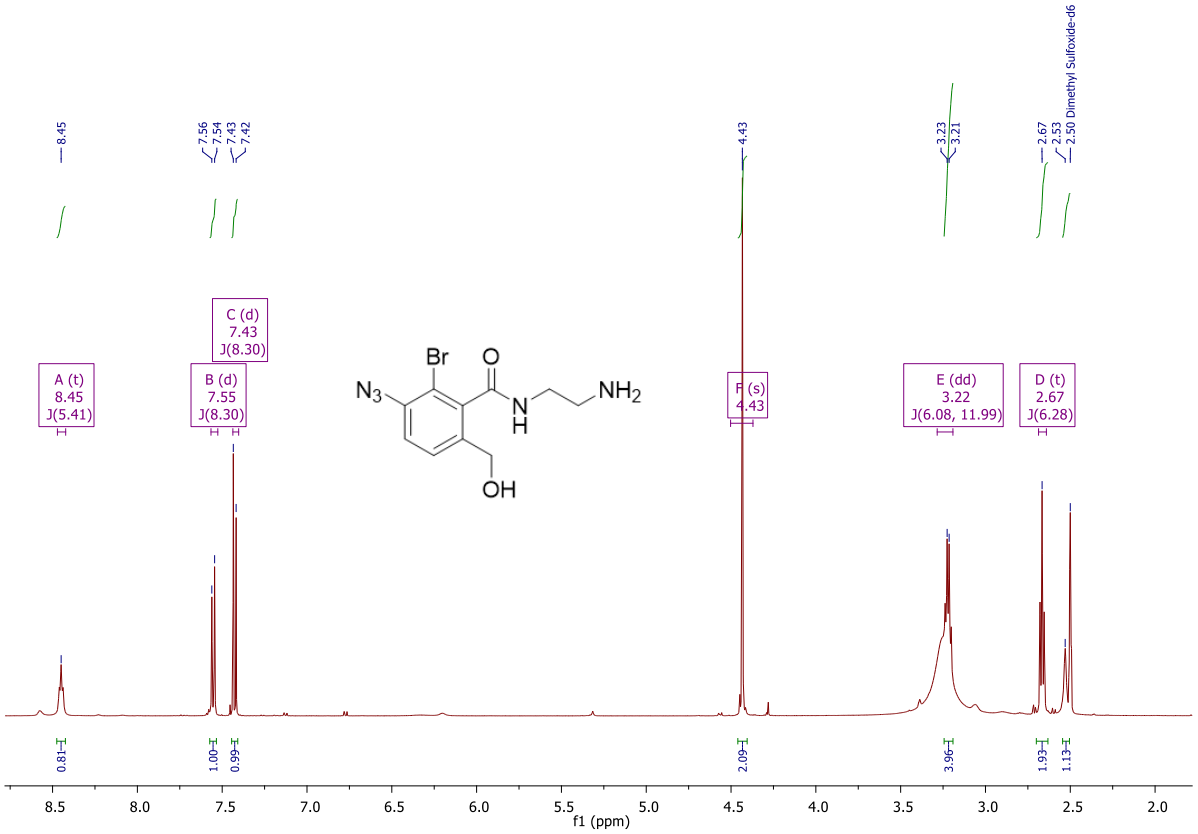


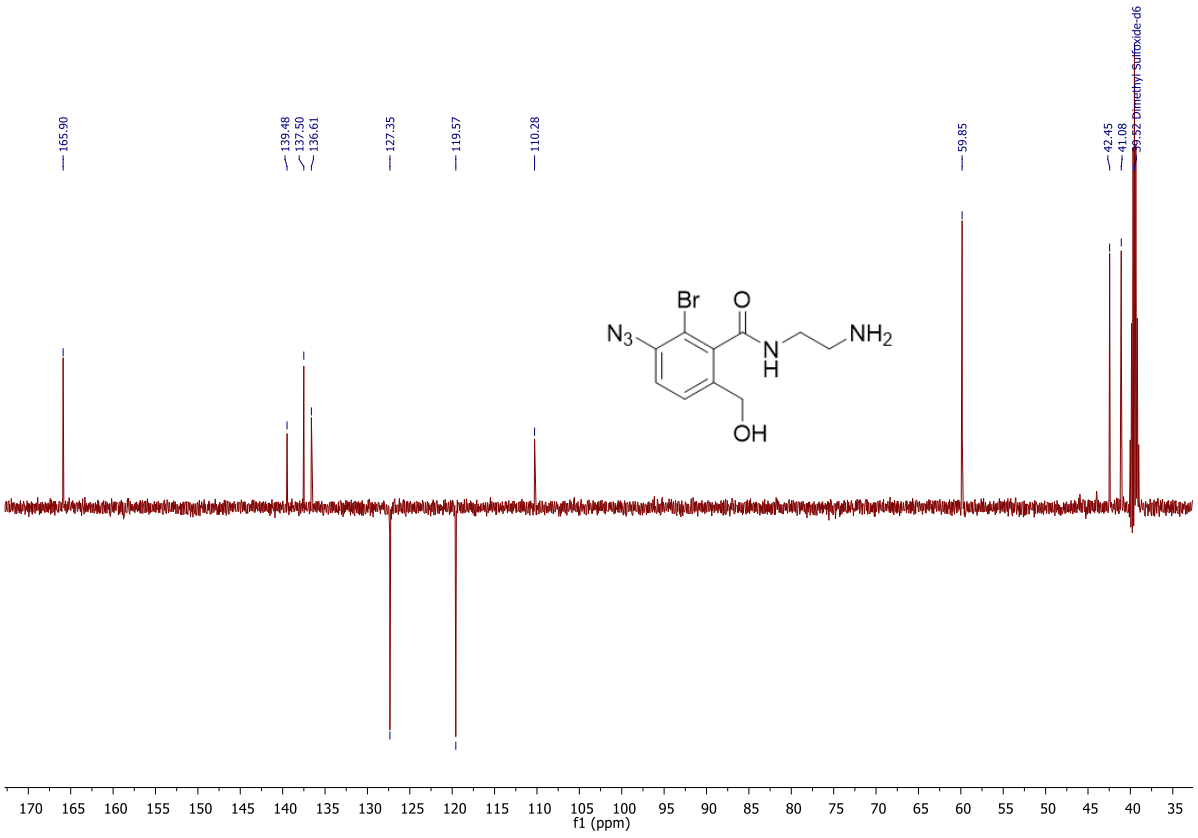
^{1}H & ^{13}C NMR spectrum of S2b



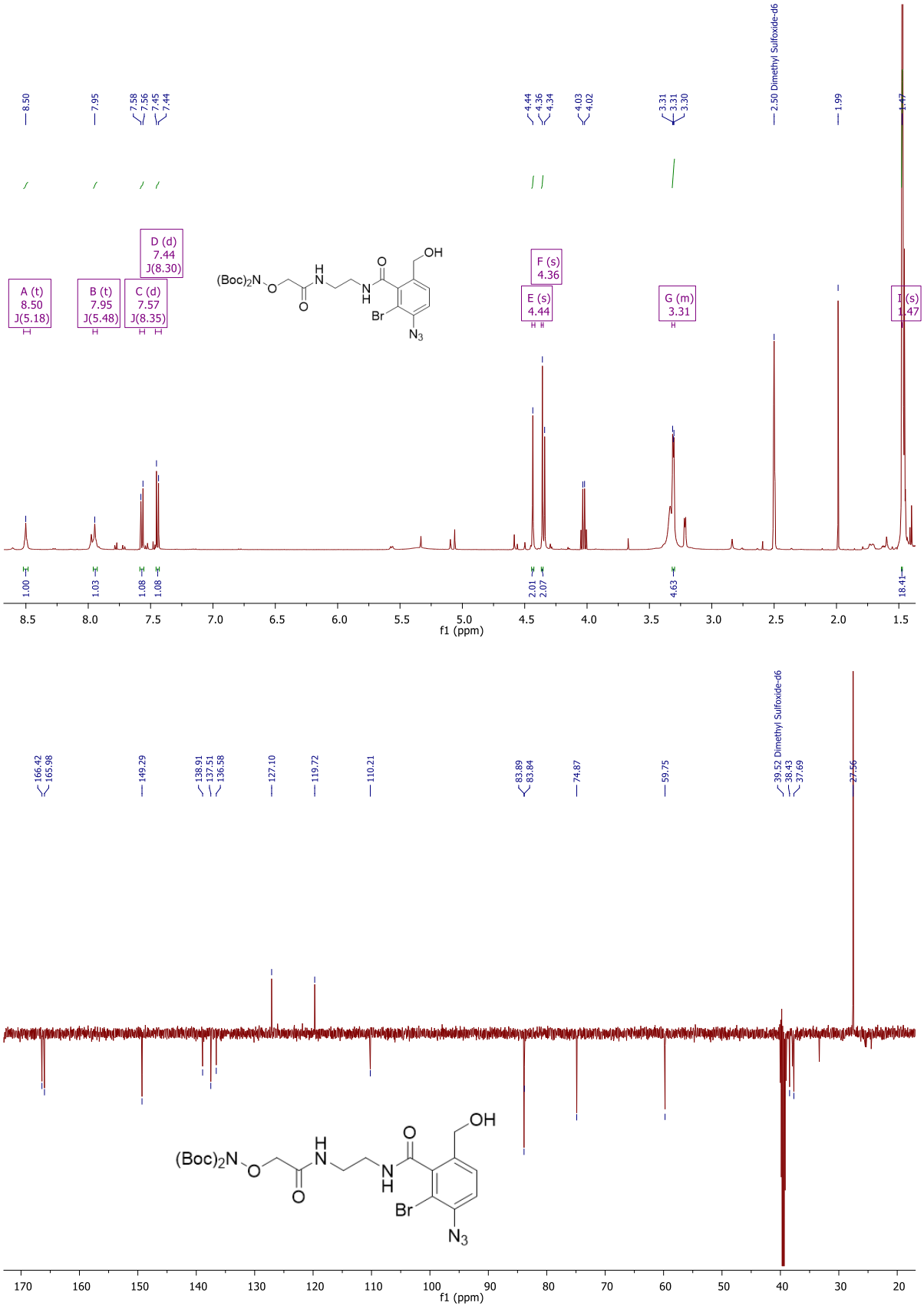


^{1}H & ^{13}C NMR spectrum of S3b

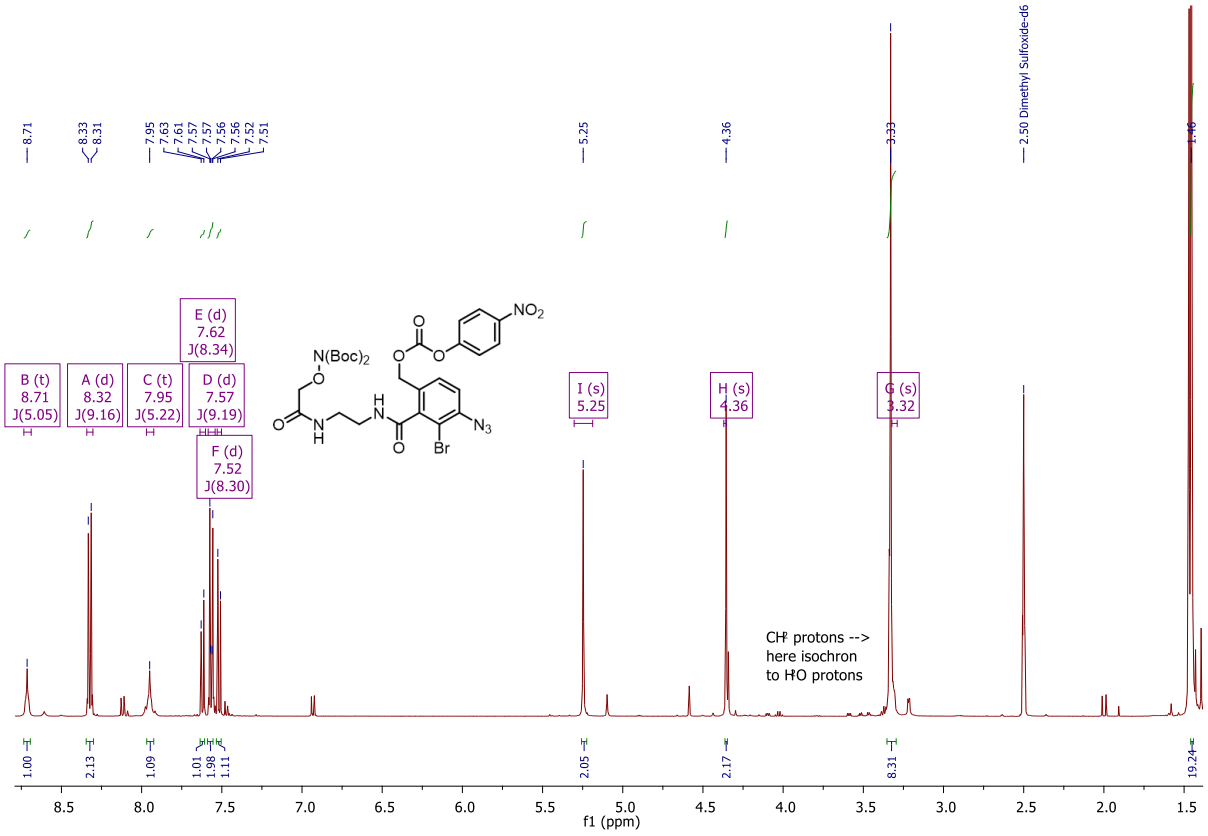


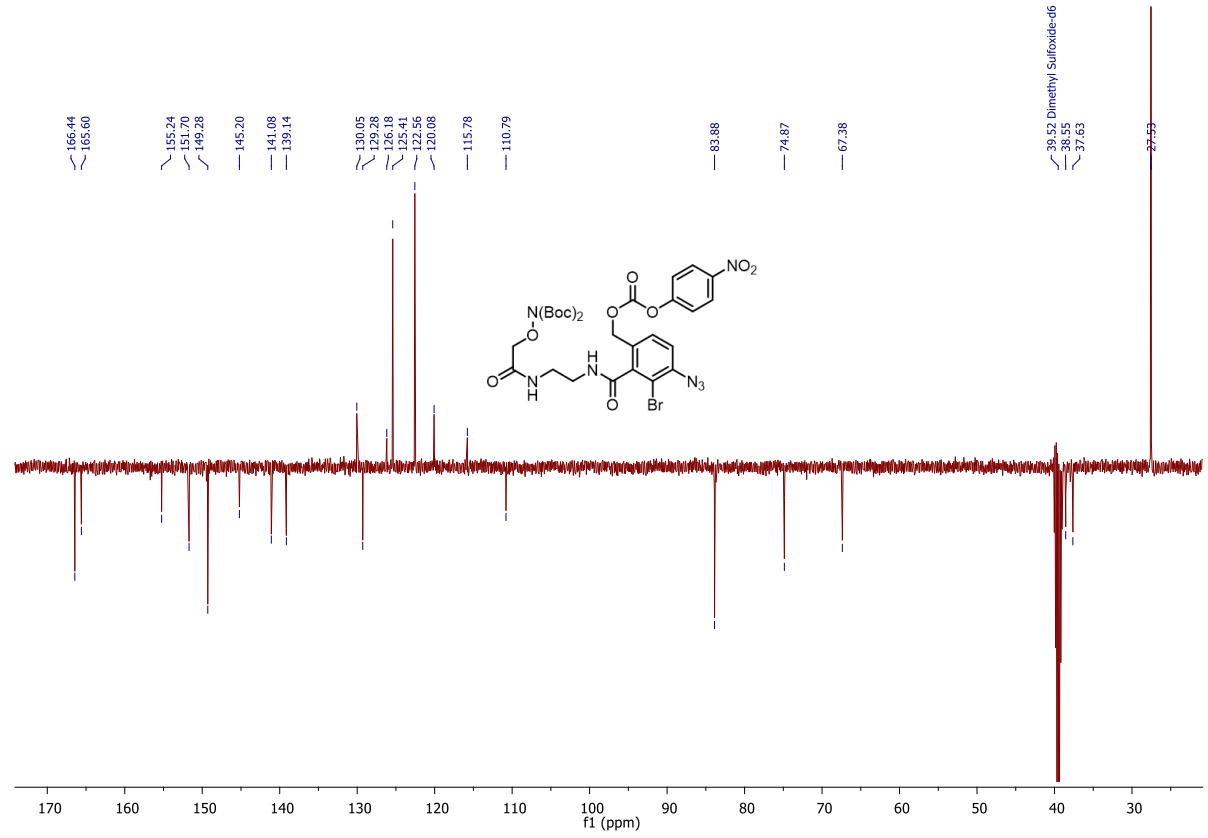


^{1}H & ^{13}C NMR spectrum of S4b

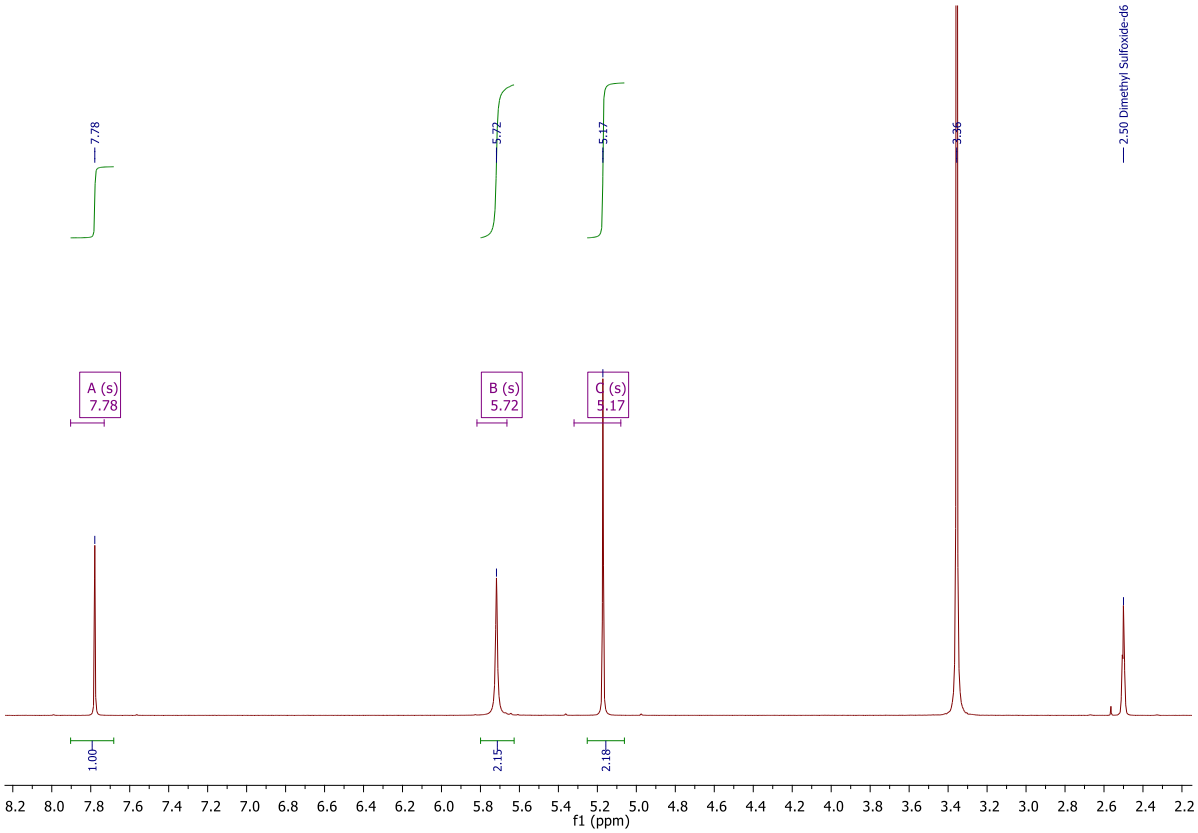


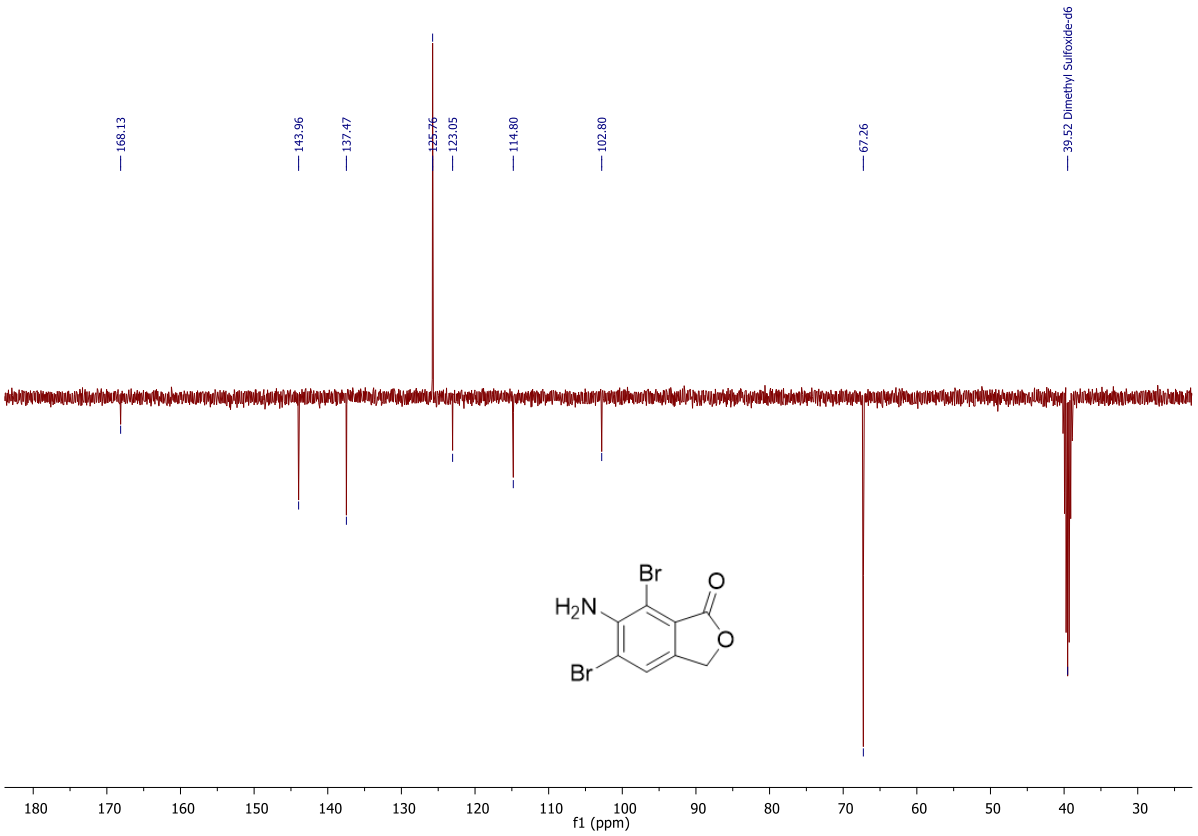
^{1}H & ^{13}C NMR spectrum of 2b





^{1}H & ^{13}C NMR spectrum of S1c





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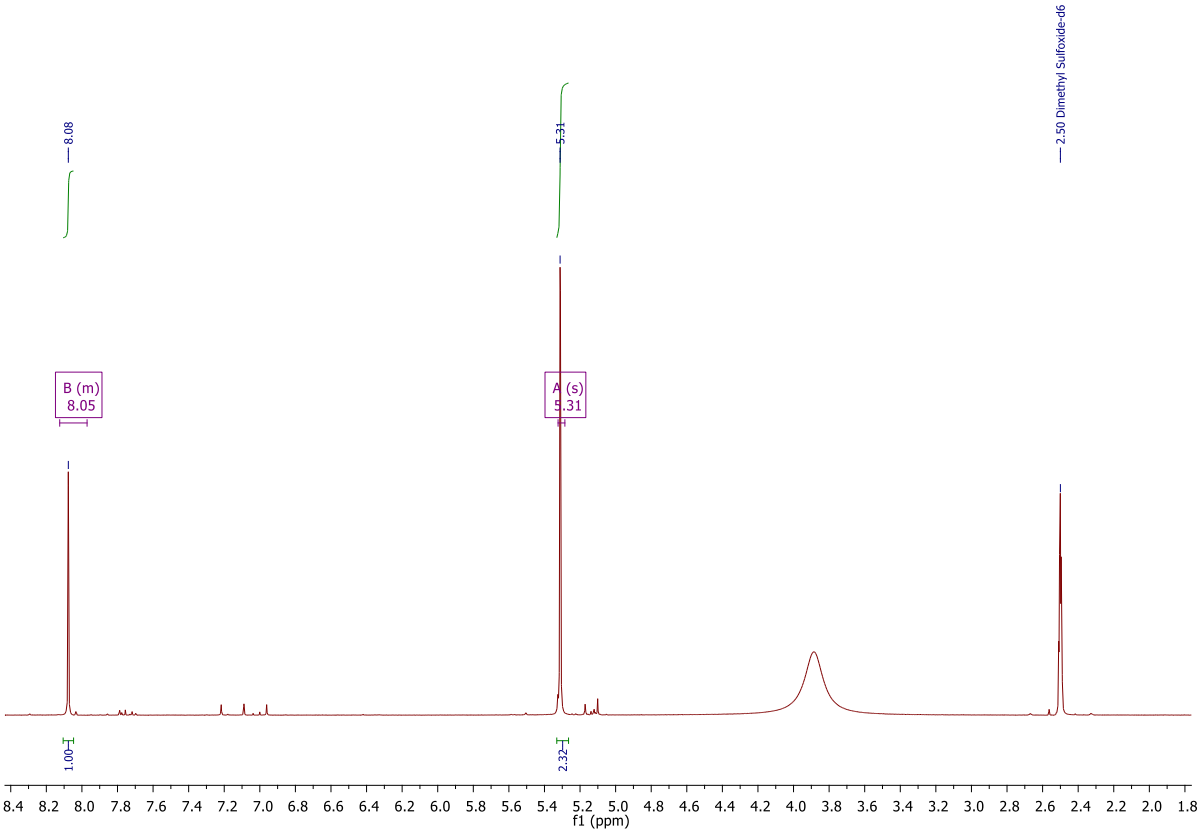
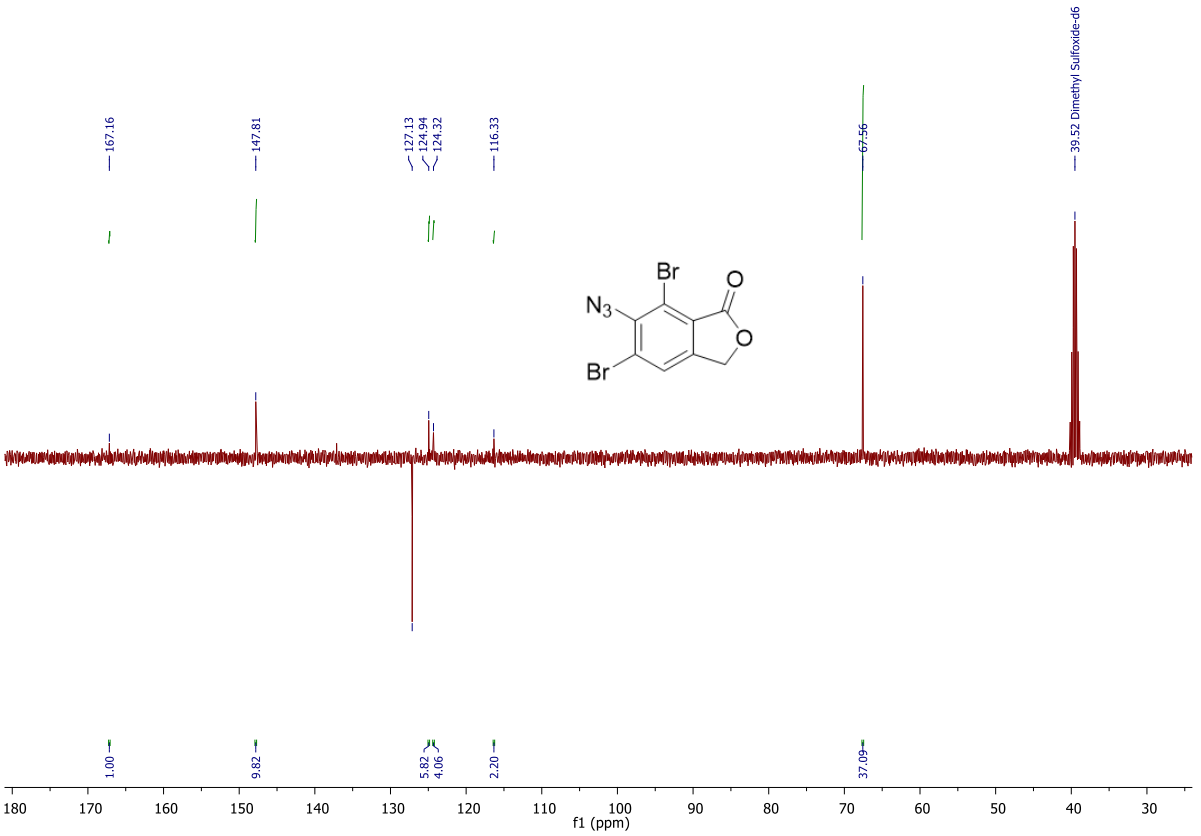
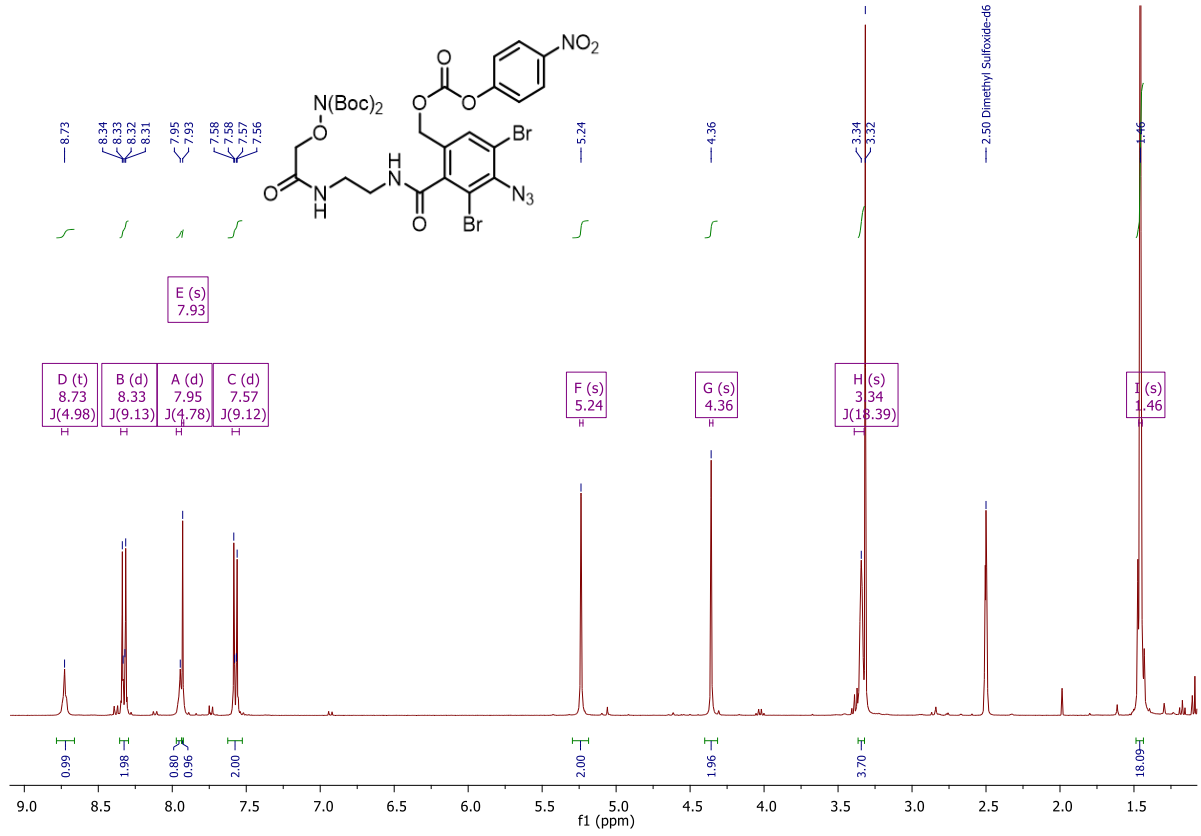
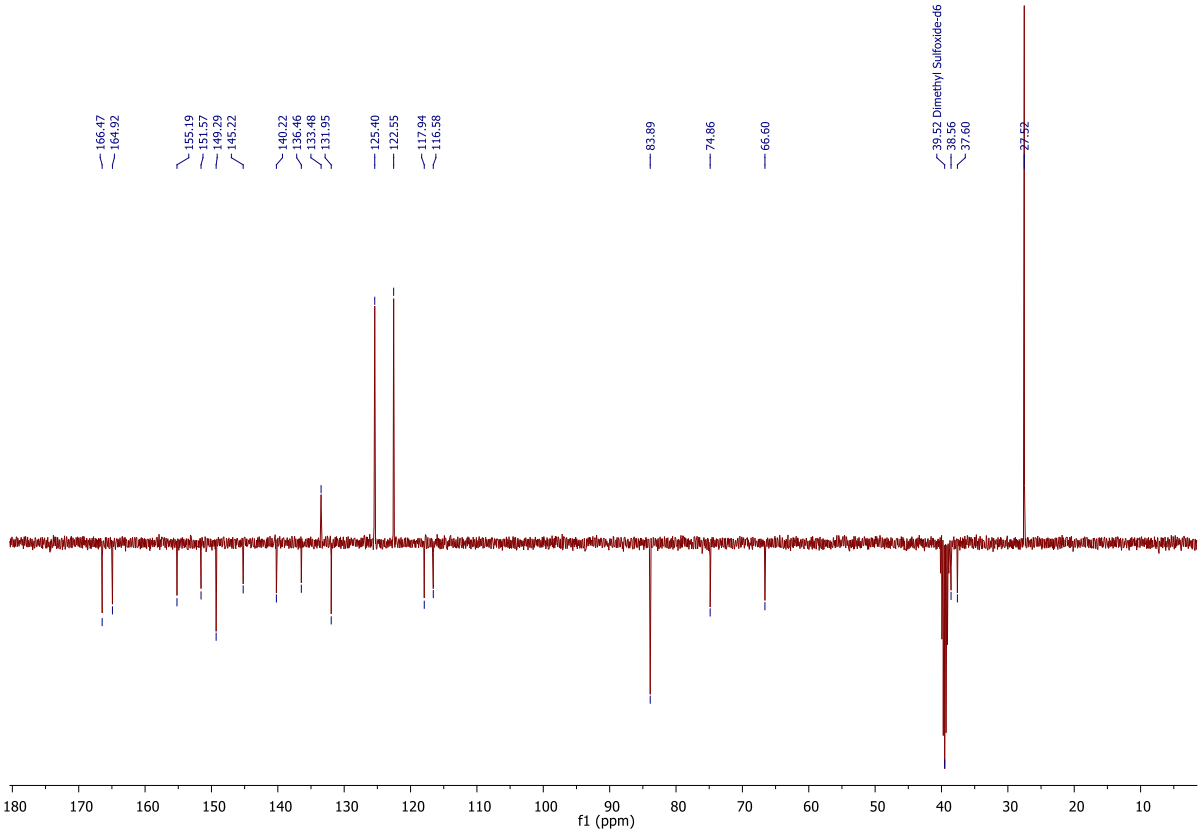




^{1}H & ^{13}C NMR spectrum of 2c





S9.2 UV-chromatograms and ESI-MS-spectra of purified peptides from the comparative linker study S3

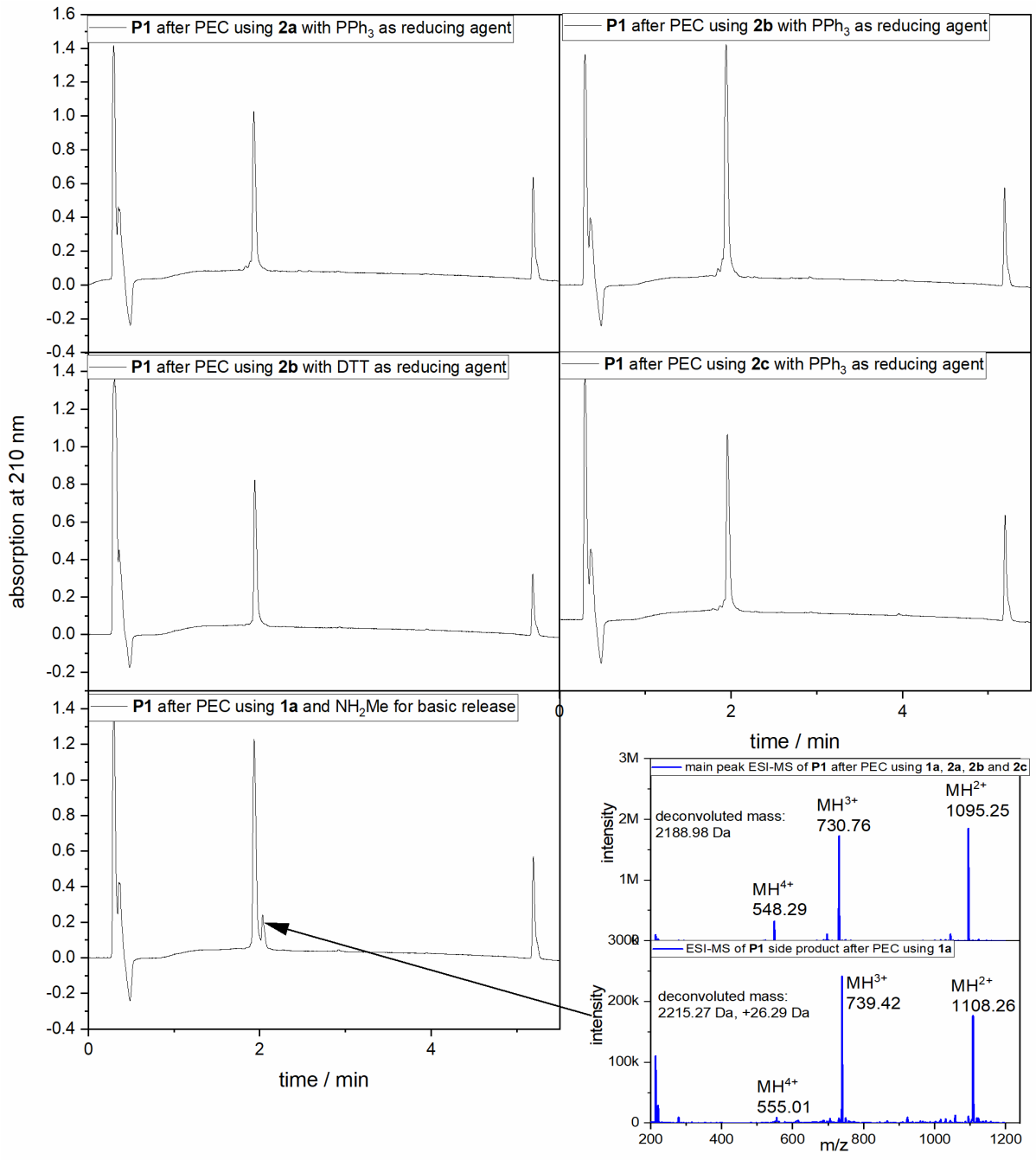


Figure S 23: UPLC-UV traces and ESI-MS signals of purified peptide product **P1**. ESI-MS of main peaks was identical in all linkers used. In case of the usage of **1a** a side product was formed with the shown ESI-MS spectrum.

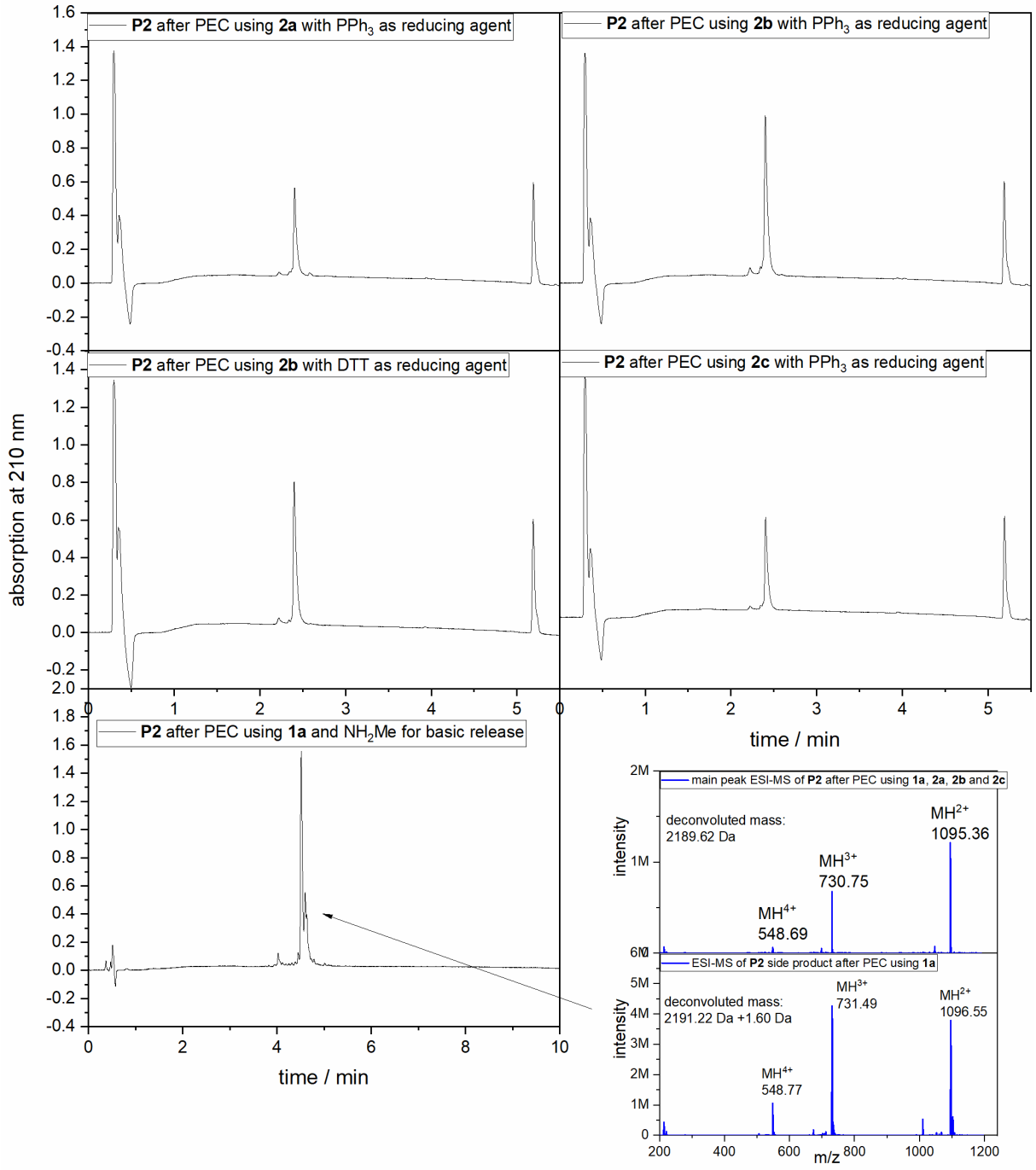


Figure S 24: UPLC-UV traces and ESI-MS signals of purified peptide product **P2**. ESI-MS of main peaks was identical in all linkers used. In case of the usage of **1a** a side product was formed with the shown ESI-MS spectrum.

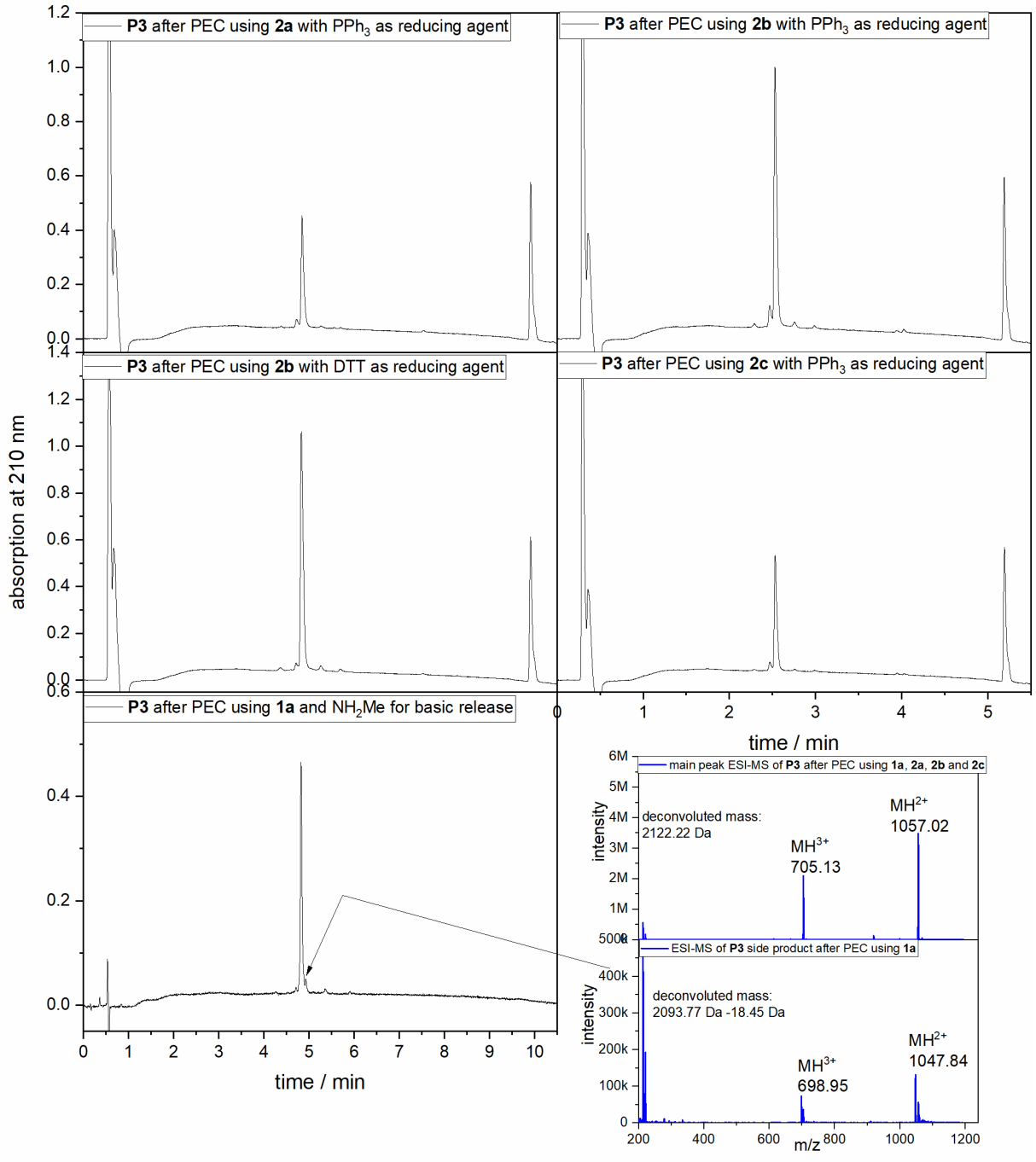


Figure S 25: UPLC-UV traces and ESI-MS Signals of purified peptide product **P3**. ESI-MS of main peaks was identical in all linkers used. In case of the usage of **1a** a side product was formed with the shown ESI-MS spectrum.

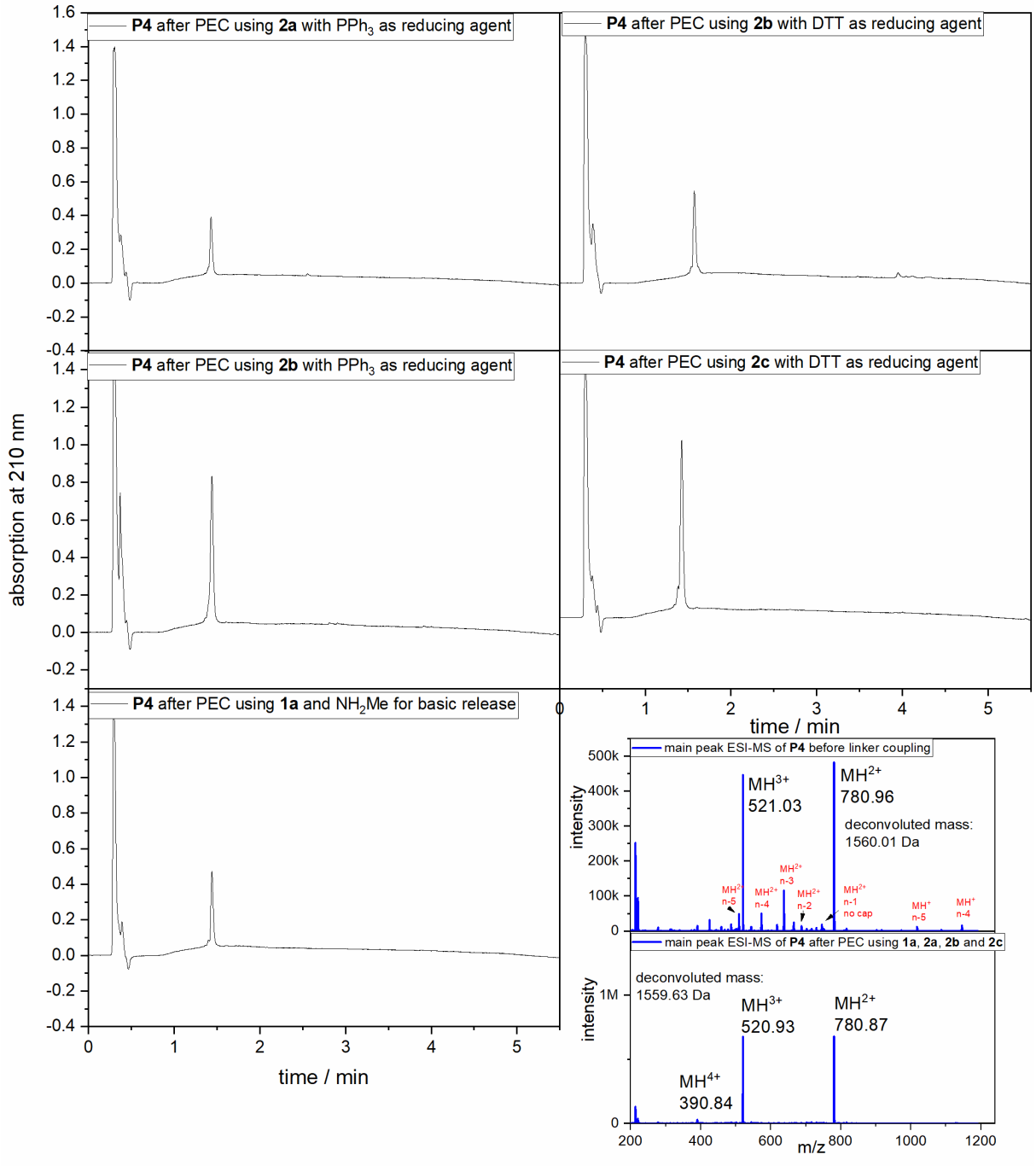
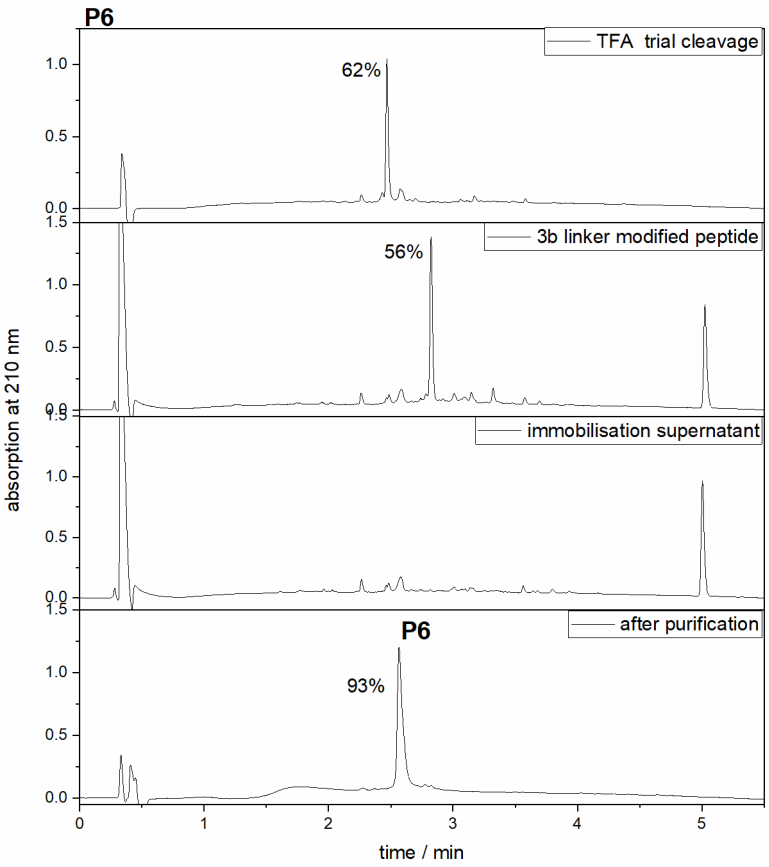
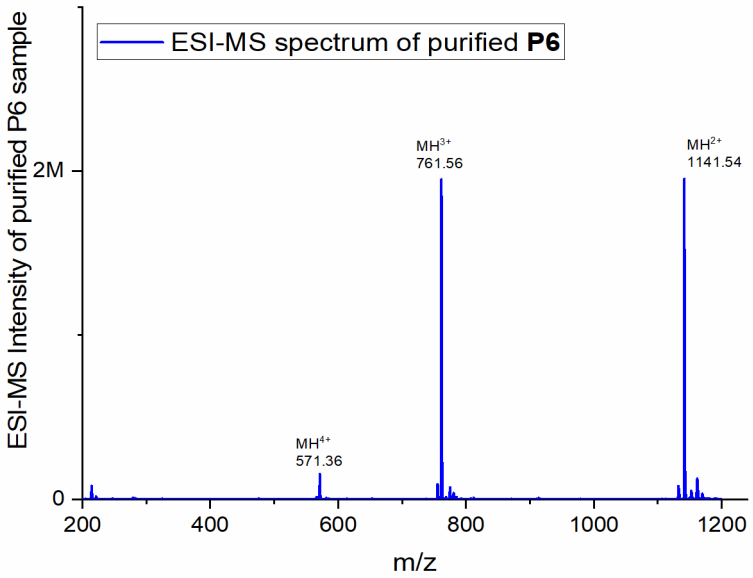
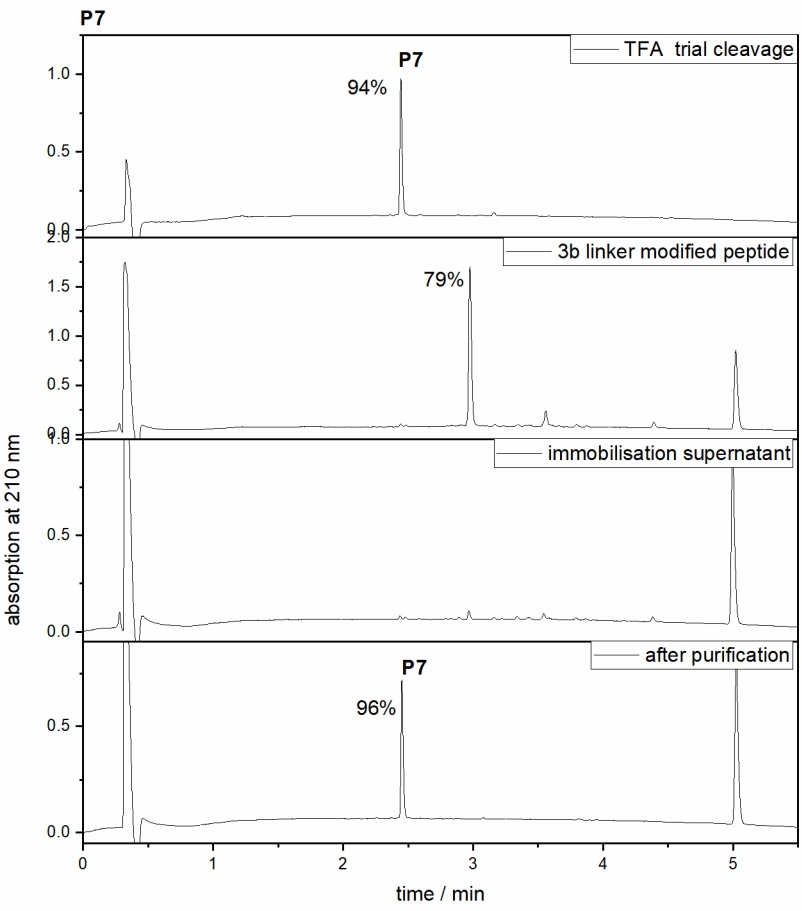


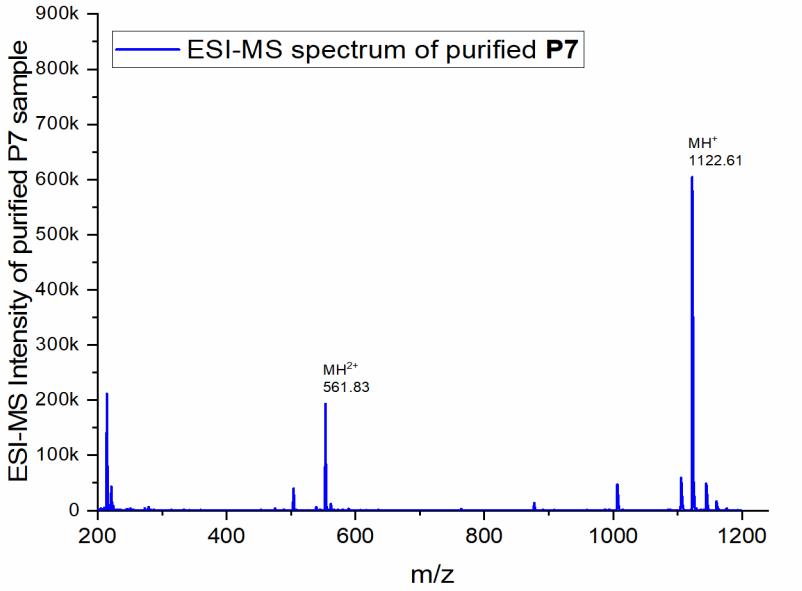
Figure S 26: UPLC-UV traces and ESI-MS Signals of purified peptide product **P4**. ESI-MS of main peaks was identical in all linkers used. Here the main peak ESI-MS spectrum of the main peak of the trial cleavage before linker coupling is shown (compare **Figure S 5** on page 30, **P4** TFA trail cleavage).

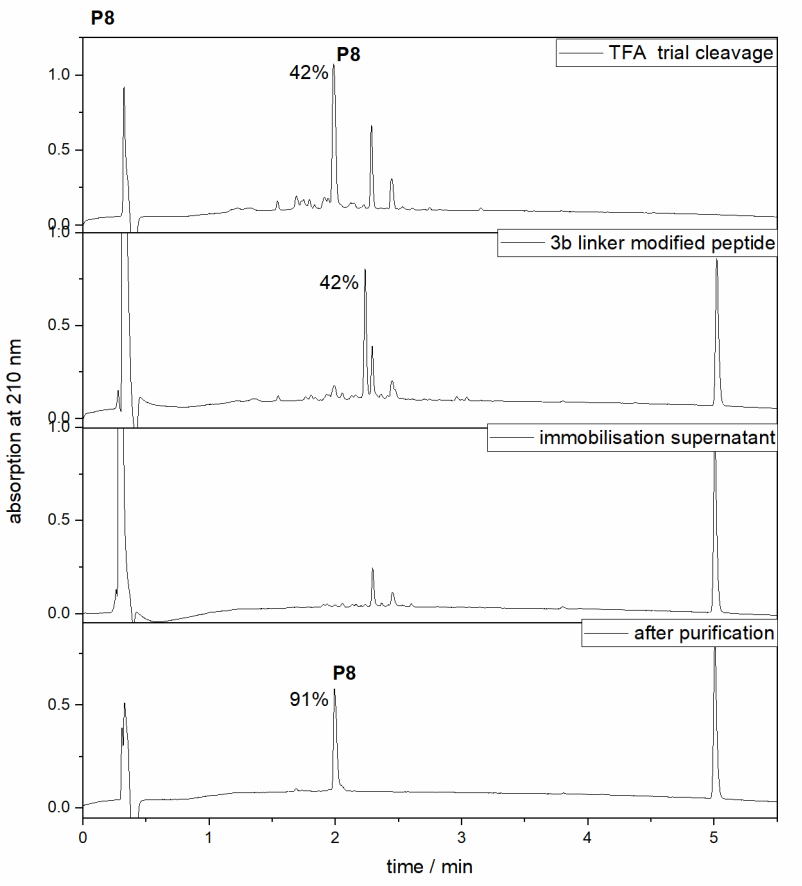
S9.3 UV-Chromatograms and ESI-MS-spectra of personalised peptide vaccine set

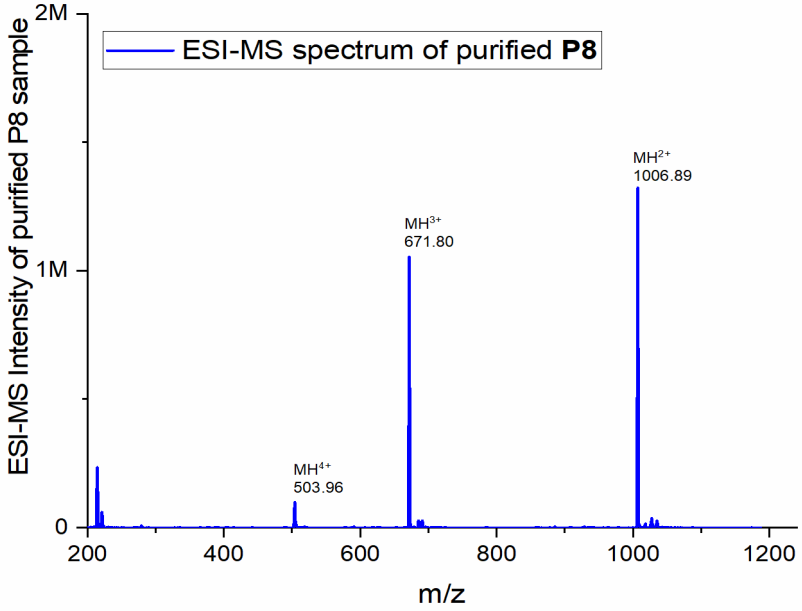


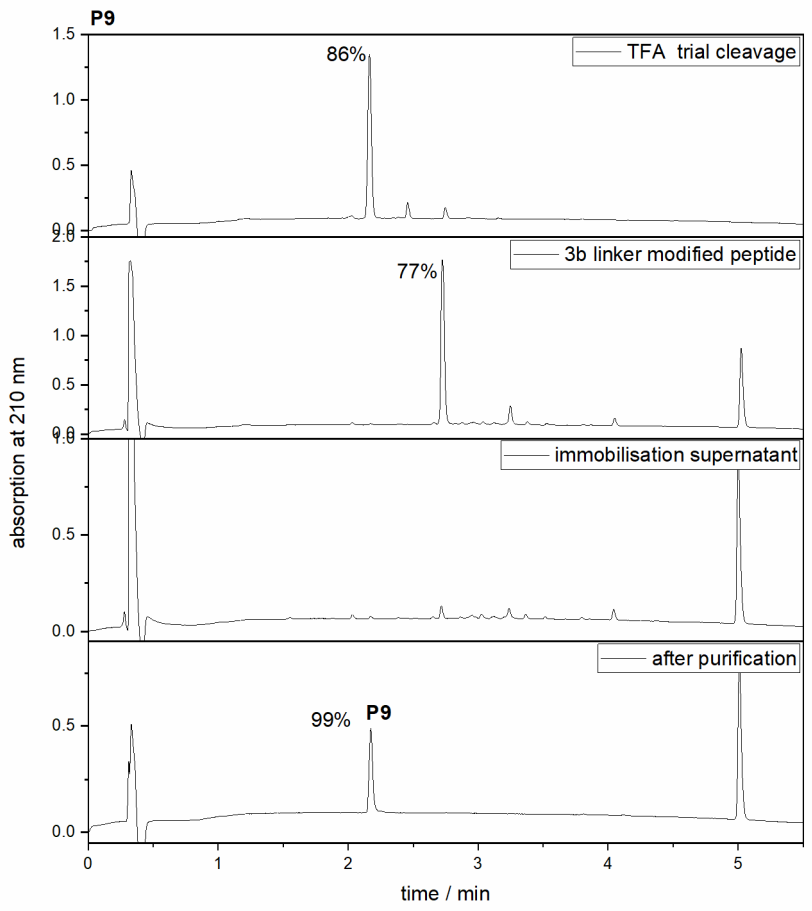


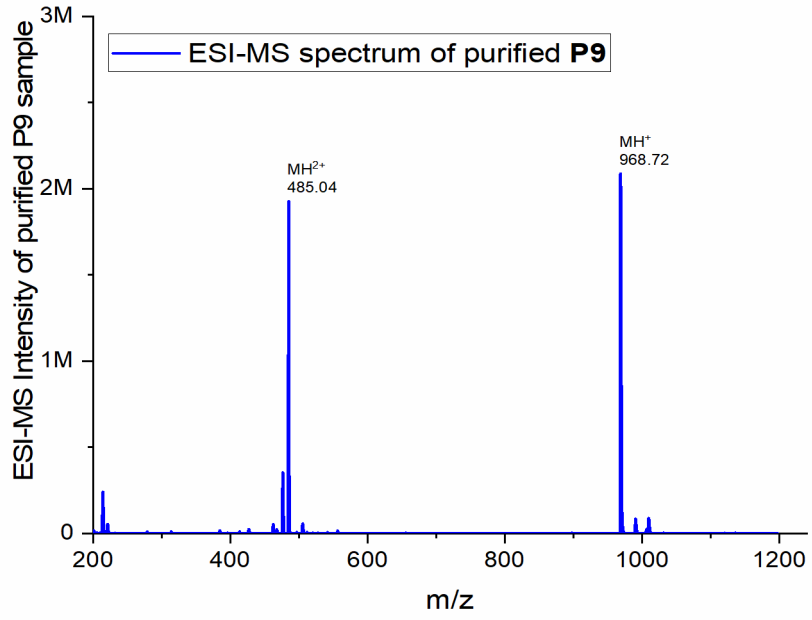

P7
TFA trial cleavage
P7
94%
3b linker modified peptide
79%
immobilisation supernatant
after purification
P7
96%
absorption at 210 nm
time / min

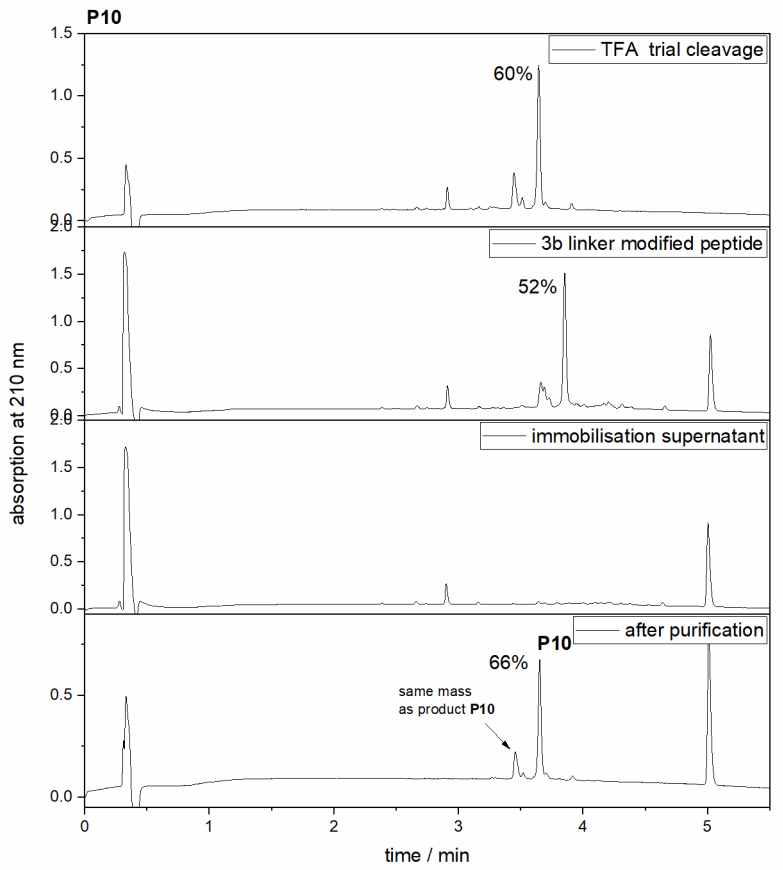

ESI-MS spectrum of purified P7
MH+
1122.61
MH2+
561.83
ESI-MS Intensity of purified P7 sample
m/z

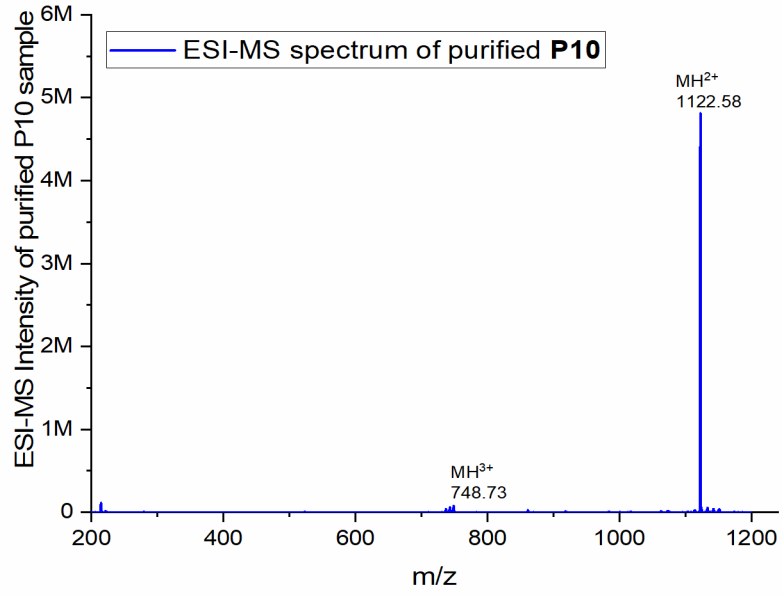

P8
TFA trial cleavage
P8
42%
3b linker modified peptide
42%
immobilisation supernatant
after purification
P8
91%
absorption at 210 nm
time / min

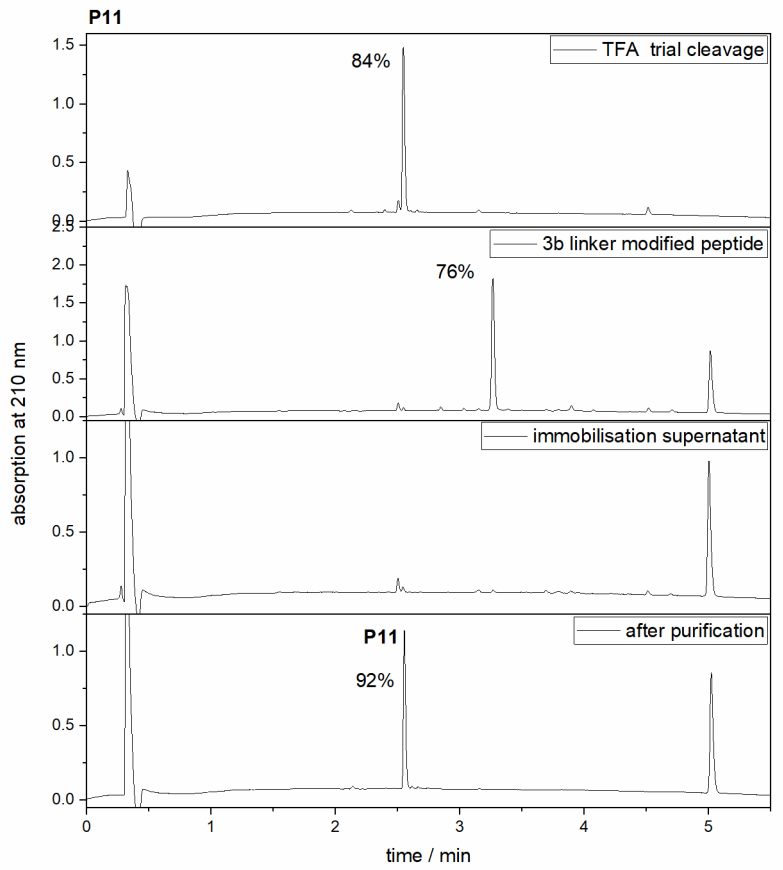

ESI-MS spectrum of purified P8
MH2+
1006.89
MH3+
671.80
MH4+
503.96
ESI-MS Intensity of purified P8 sample
m/z

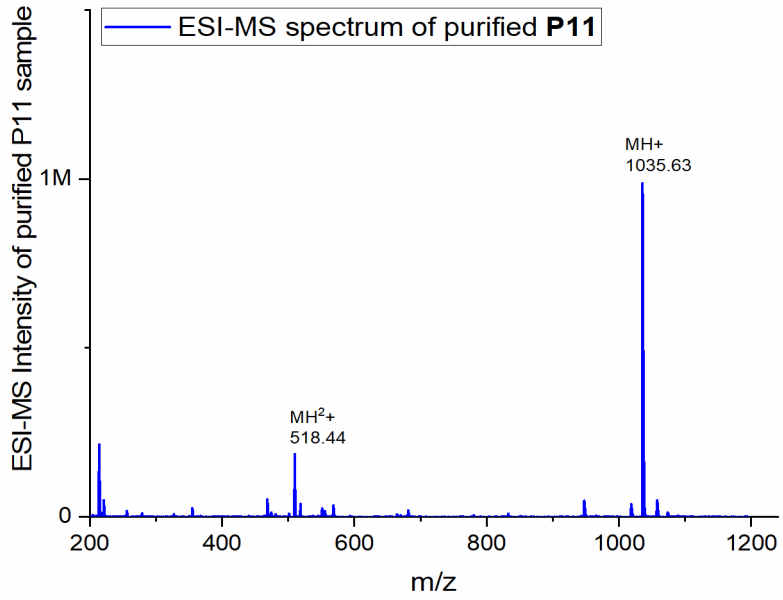

P9
TFA trial cleavage
86%
3b linker modified peptide
77%
immobilisation supernatant
after purification
99% P9
absorption at 210 nm
time / min

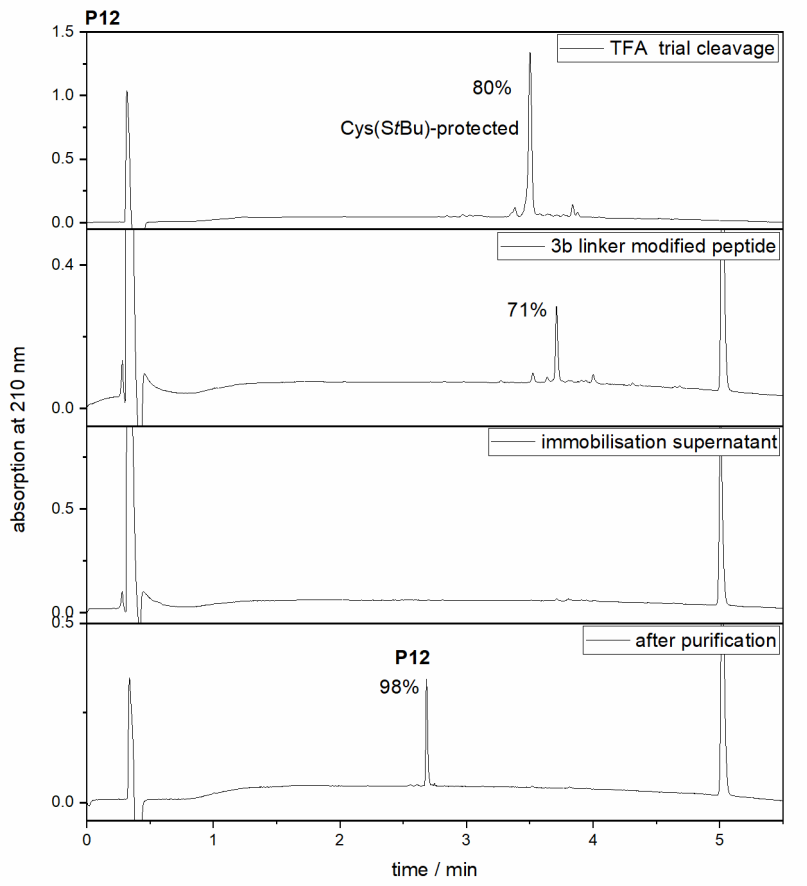

ESI-MS spectrum of purified P9
MH2+
485.04
MH+
968.72
ESI-MS Intensity of purified P9 sample
m/z

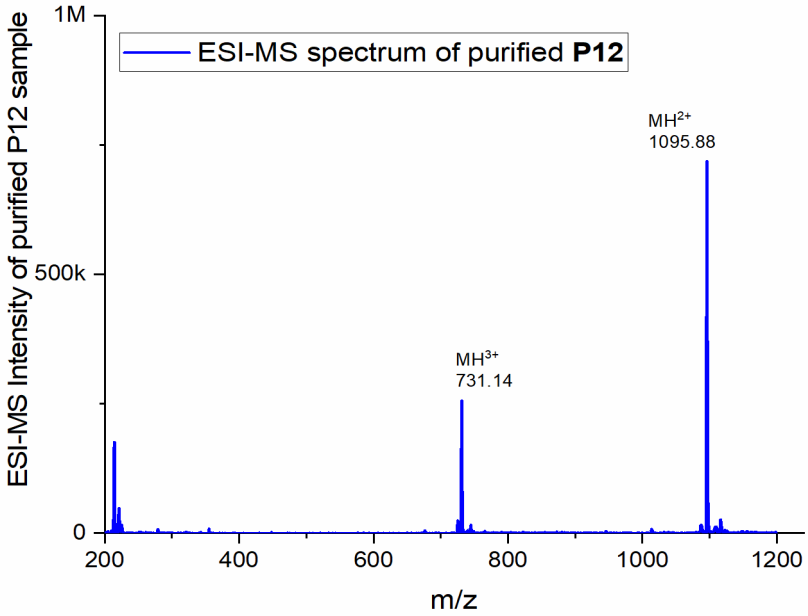

P10
TFA trial cleavage
60%
3b linker modified peptide
52%
immobilisation supernatant
after purification
P10
66%
same mass
as product P10
absorption at 210 nm
time / min

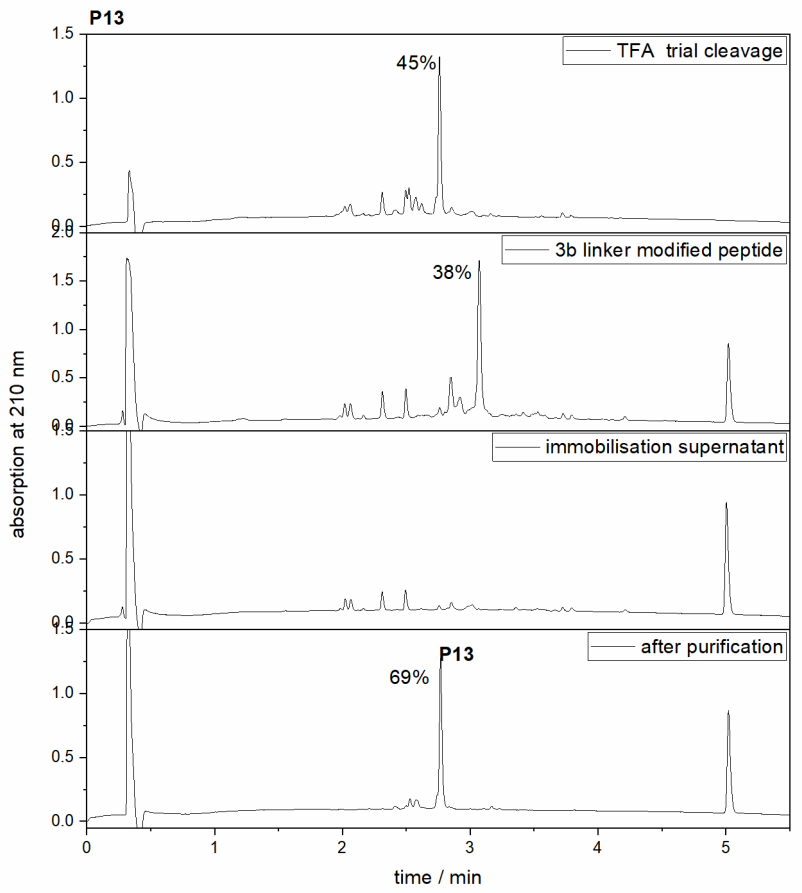

ESI-MS spectrum of purified P10
MH2+
1122.58
MH3+
748.73
ESI-MS Intensity of purified P10 sample
m/z

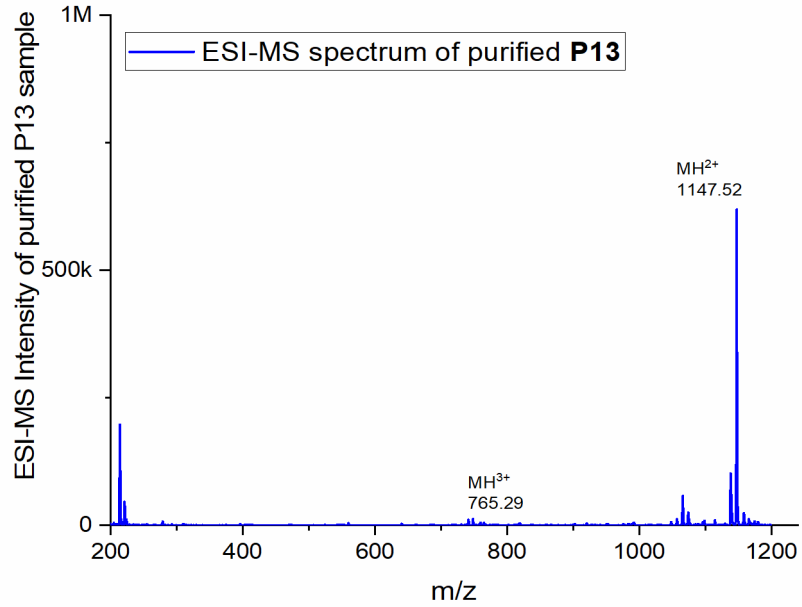

P11
TFA trial cleavage
84%
3b linker modified peptide
76%
immobilisation supernatant
P11
92%
after purification
absorption at 210 nm
time / min

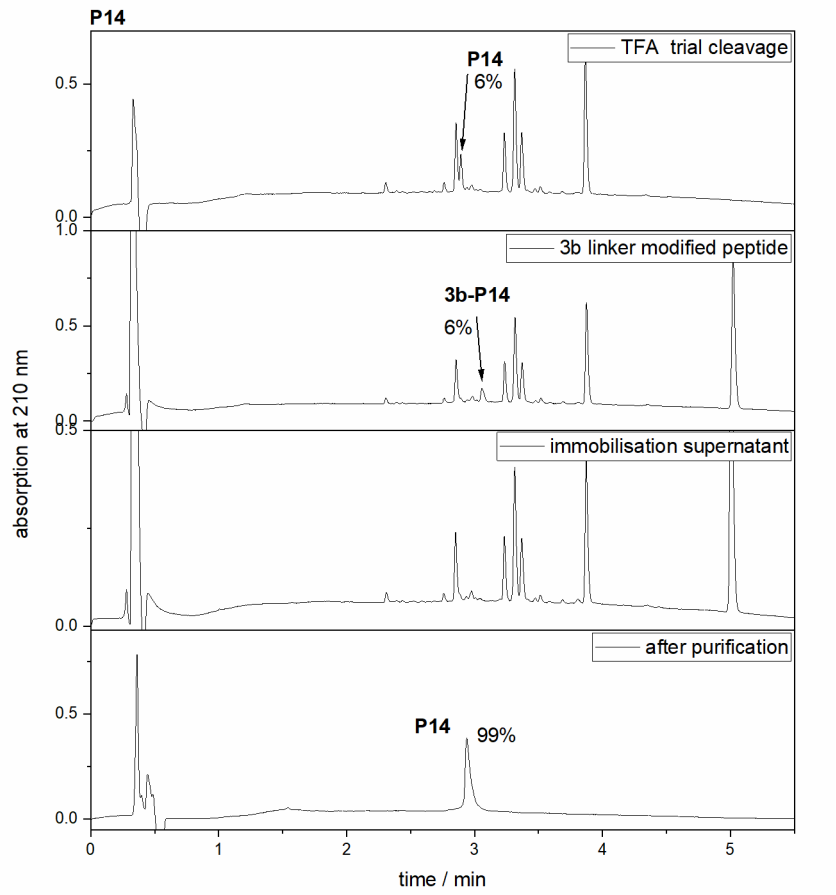

ESI-MS spectrum of purified P11
MH+
1035.63
MH2+
518.44
ESI-MS Intensity of purified P11 sample
m/z

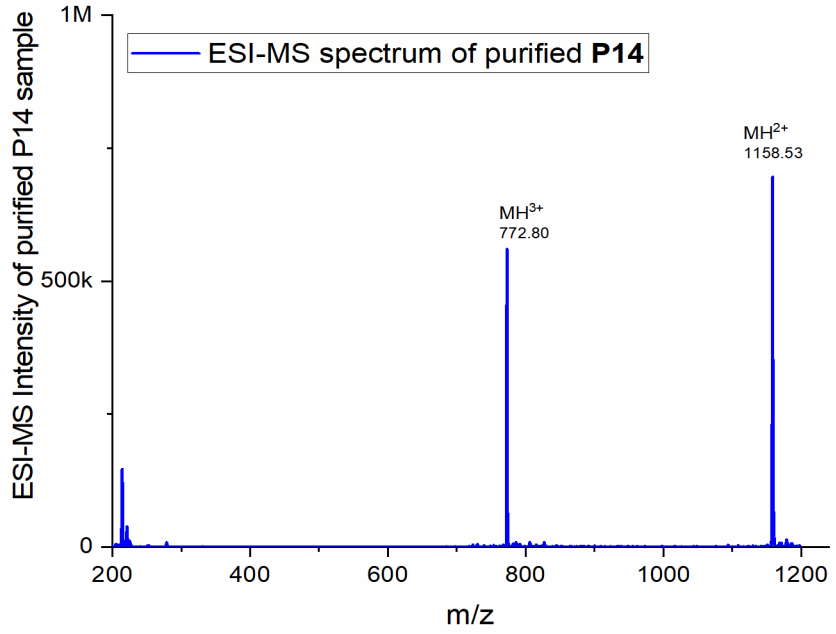

P12
TFA trial cleavage
80%
Cys(StBu)-protected
3b linker modified peptide
71%
immobilisation supernatant
after purification
P12
98%
absorption at 210 nm
time / min

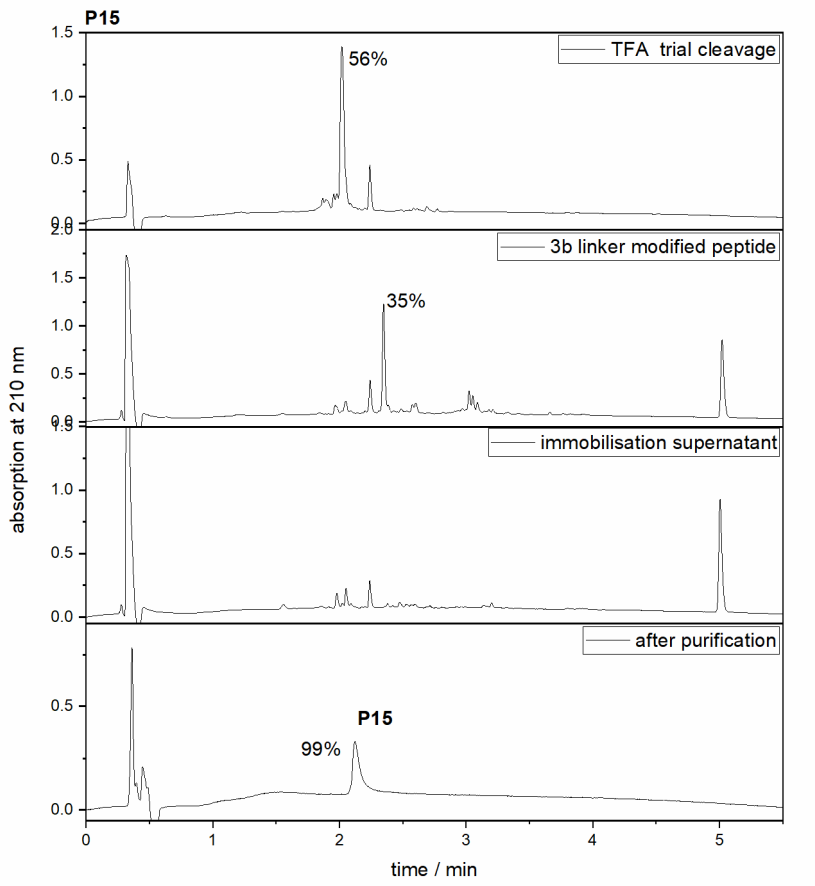

ESI-MS spectrum of purified P12
MH2+
1095.88
MH3+
731.14
ESI-MS Intensity of purified P12 sample
m/z

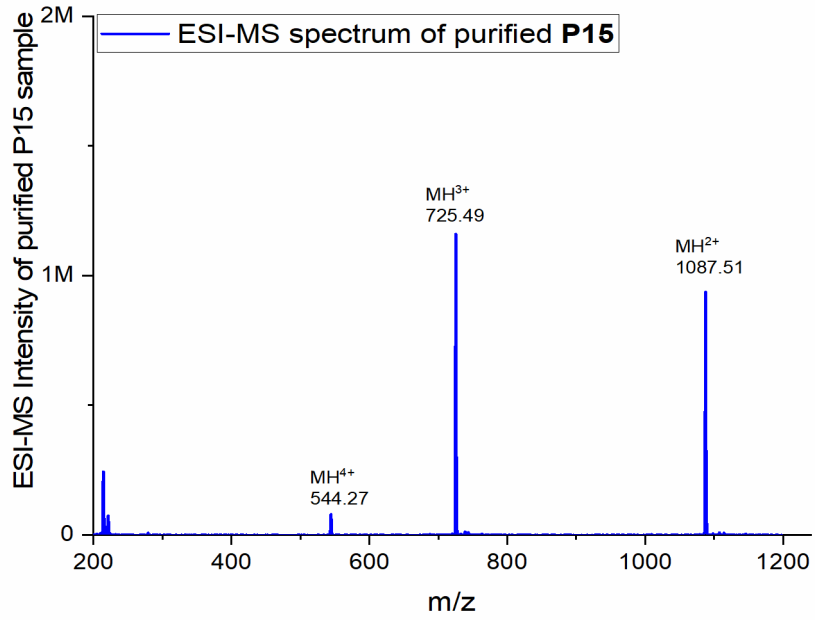

P13
TFA trial cleavage
45%
3b linker modified peptide
38%
immobilisation supernatant
after purification
P13
69%
absorption at 210 nm
time / min

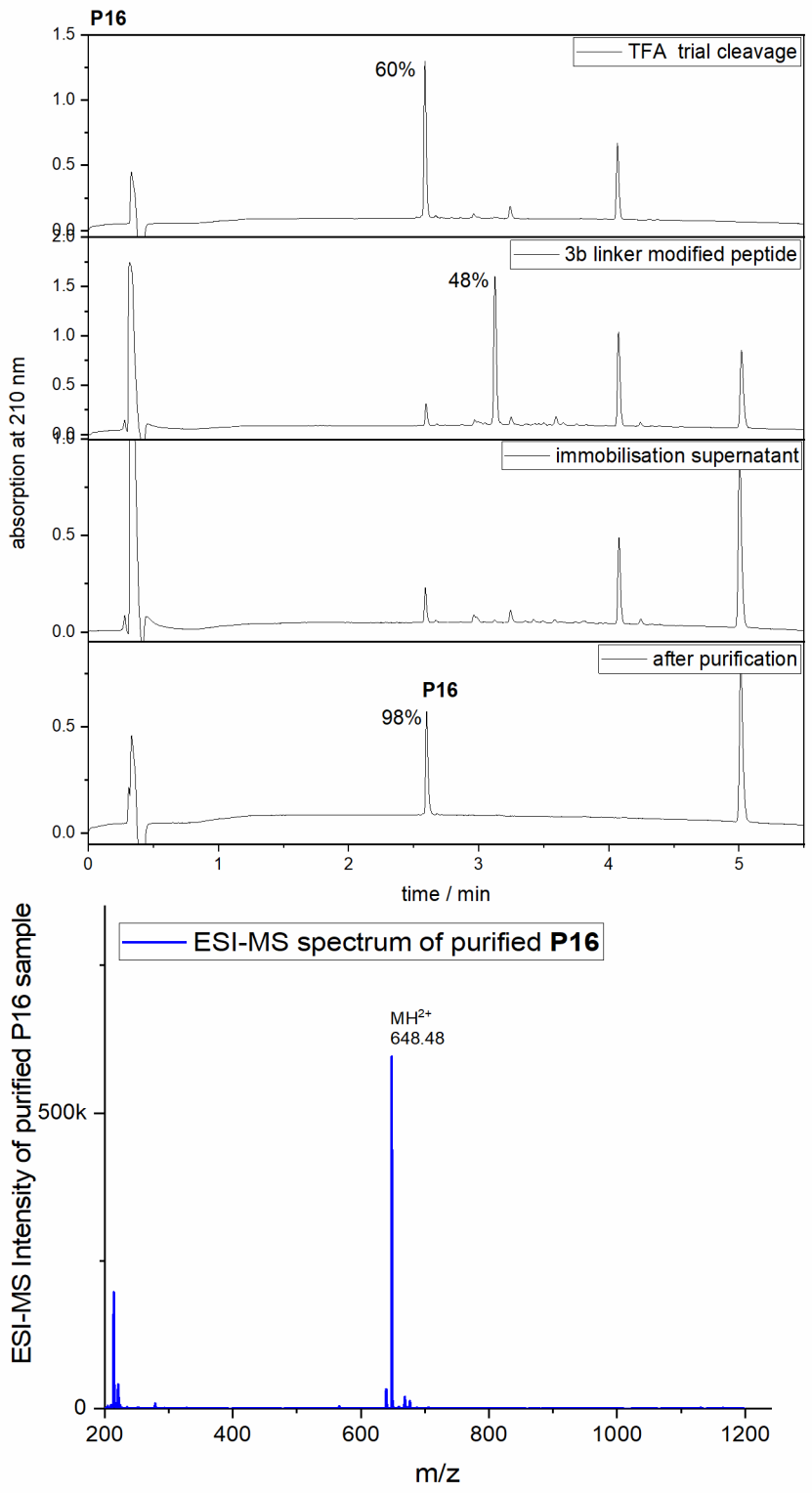

ESI-MS spectrum of purified P13
MH2+
1147.52
MH3+
765.29
ESI-MS Intensity of purified P13 sample
m/z

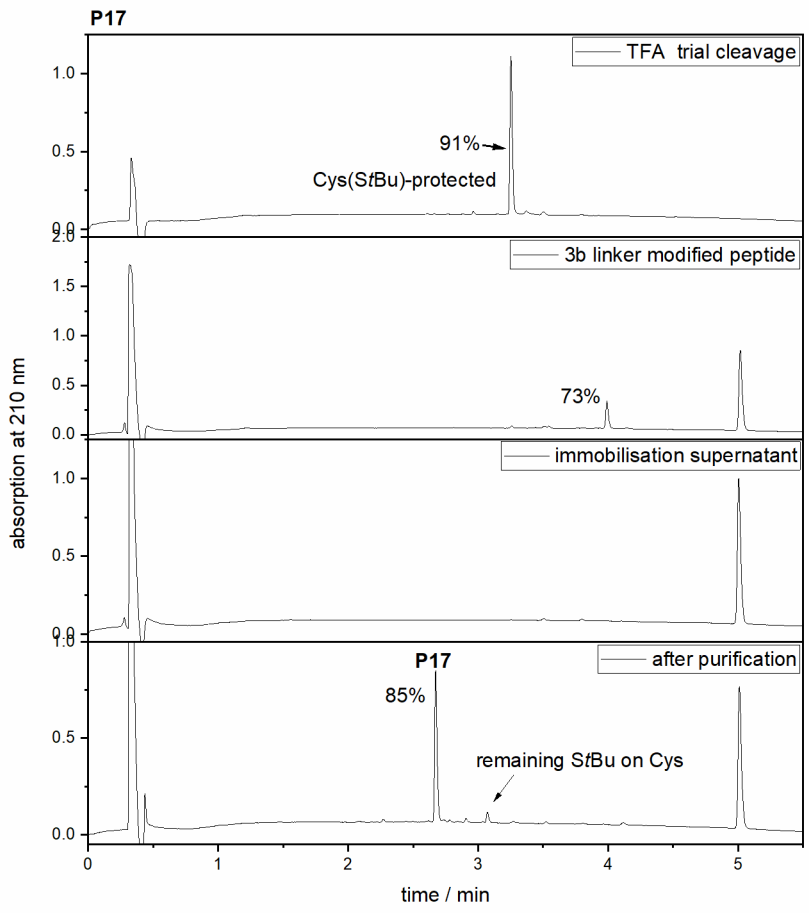

P14
TFA trial cleavage
P14
6%
3b linker modified peptide
3b-P14
6%
immobilisation supernatant
after purification
P14
99%
absorption at 210 nm
time / min

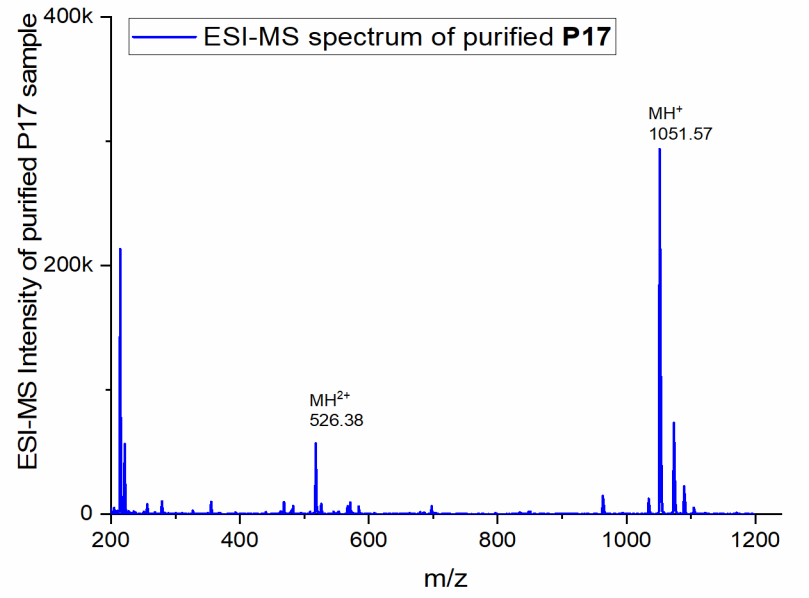

ESI-MS spectrum of purified P14
MH2+
1158.53
MH3+
772.80
ESI-MS Intensity of purified P14 sample
m/z

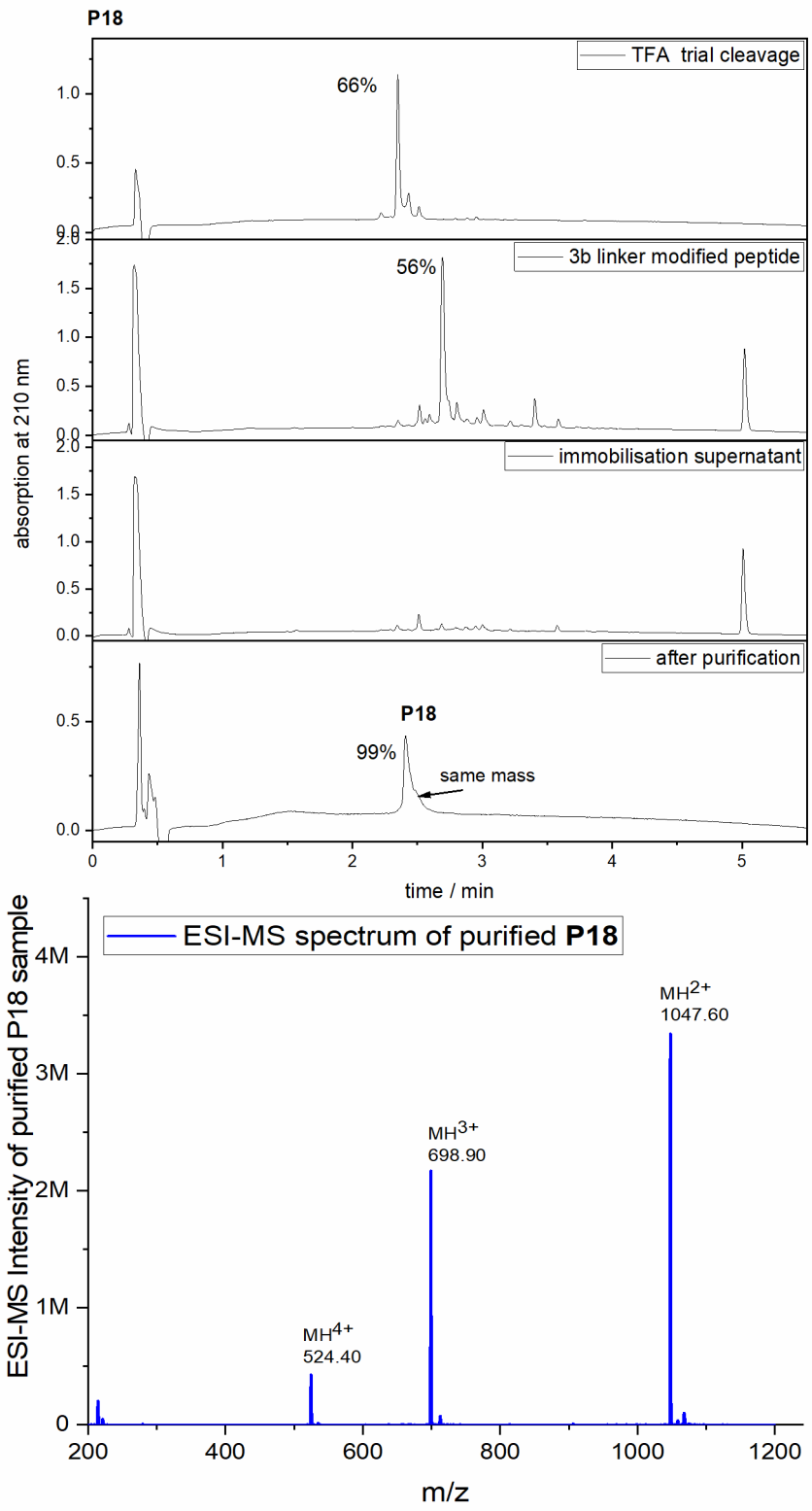

P15
TFA trial cleavage
56%
3b linker modified peptide
35%
immobilisation supernatant
after purification
P15
99%
absorption at 210 nm
time / min

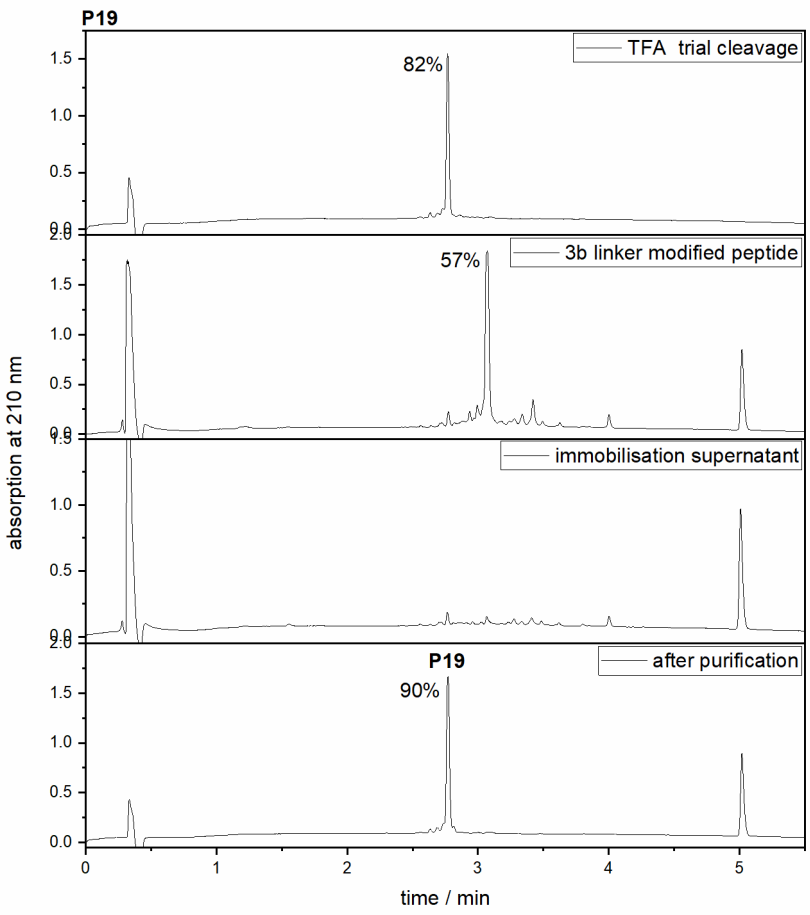

ESI-MS spectrum of purified P15
MH3+
725.49
MH2+
1087.51
MH4+
544.27
ESI-MS Intensity of purified P15 sample
m/z

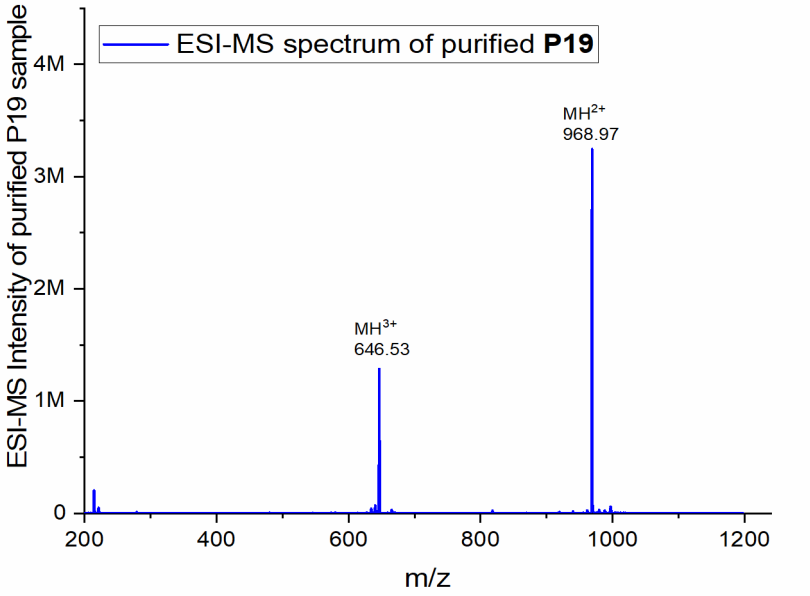

P16
TFA trial cleavage
60%
3b linker modified peptide
48%
immobilisation supernatant
after purification
P16
98%
absorption at 210 nm
time / min
ESI-MS spectrum of purified P16
MH2+
648.48
ESI-MS Intensity of purified P16 sample
500k
0
200
400
600
800
1000
1200
m/z

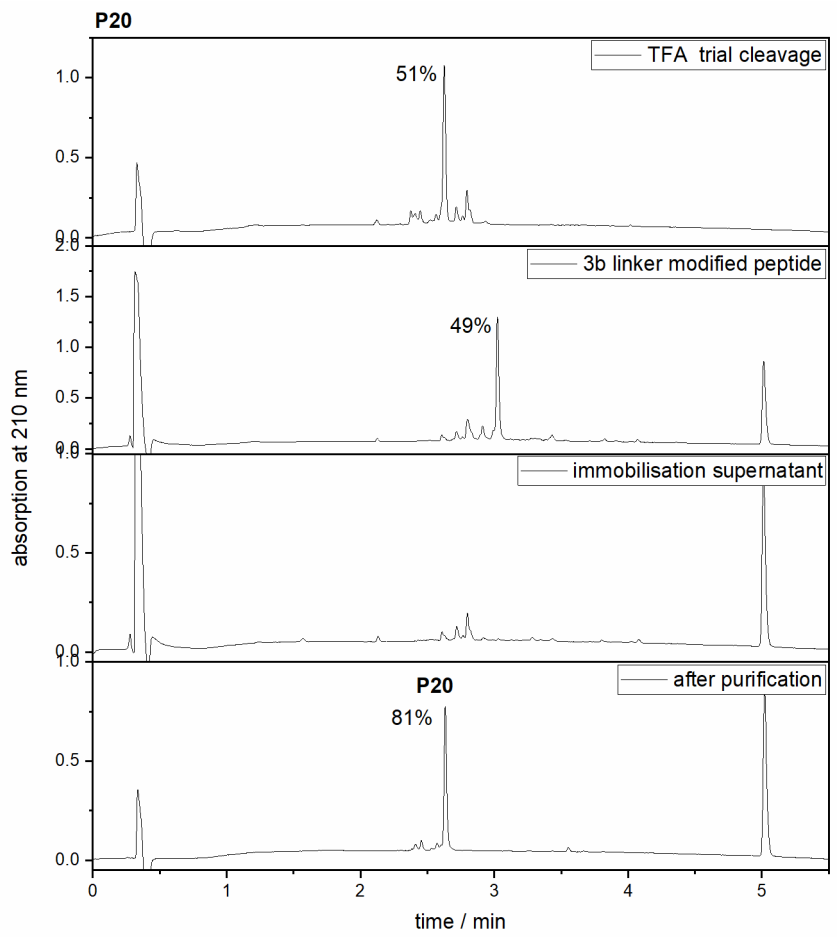

P17
TFA trial cleavage
91%
Cys(StBu)-protected
3b linker modified peptide
73%
immobilisation supernatant
P17
85%
remaining StBu on Cys
after purification
absorption at 210 nm
time / min

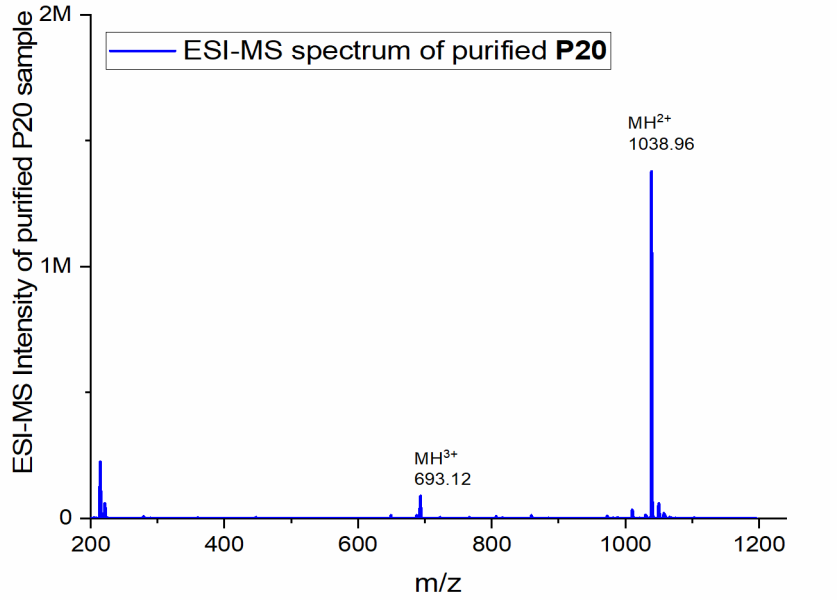

ESI-MS spectrum of purified P17
MH+
1051.57
MH2+
526.38
ESI-MS Intensity of purified P17 sample
m/z

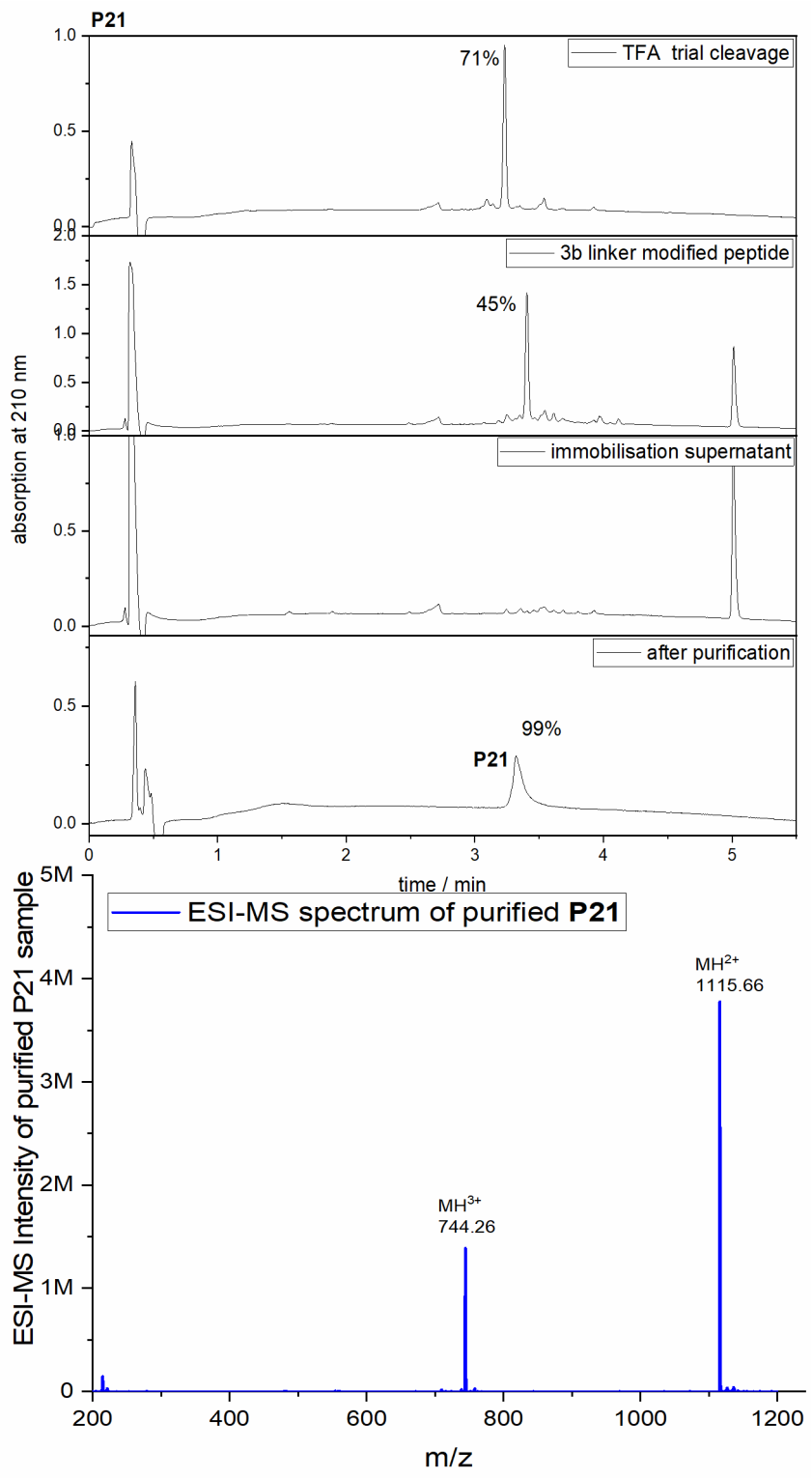

P18
TFA trial cleavage
66%
3b linker modified peptide
56%
immobilisation supernatant
after purification
P18
99%
same mass
absorption at 210 nm
time / min
ESI-MS spectrum of purified P18
ESI-MS Intensity of purified P18 sample
MH2+
1047.60
MH3+
698.90
MH4+
524.40
m/z

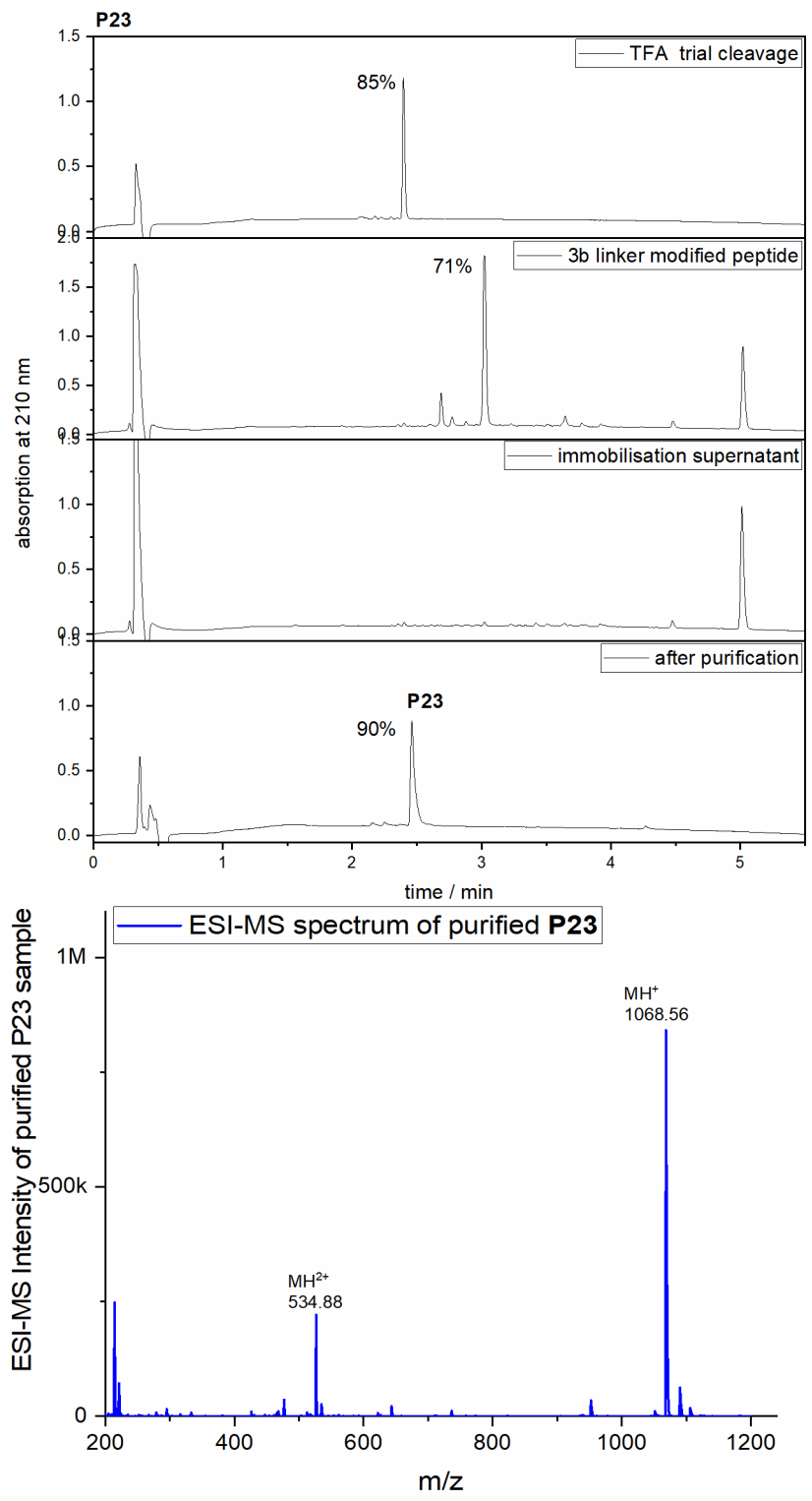

P19
TFA trial cleavage
82%
3b linker modified peptide
57%
immobilisation supernatant
P19
after purification
90%
absorption at 210 nm
time / min

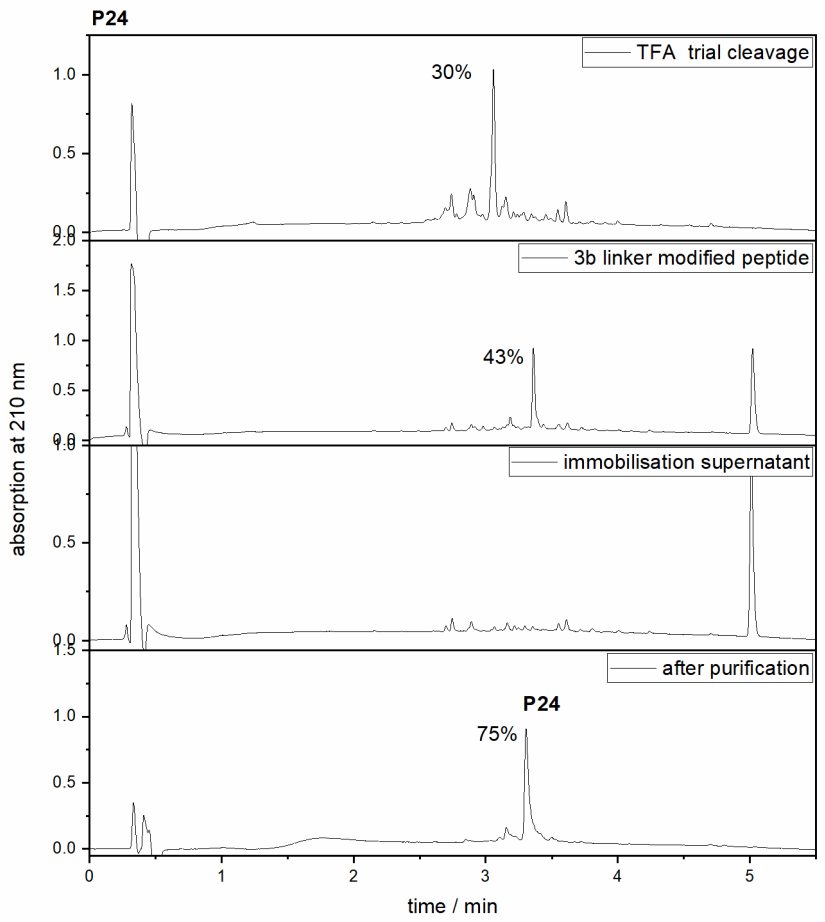

ESI-MS spectrum of purified P19
MH2+
968.97
MH3+
646.53
ESI-MS Intensity of purified P19 sample
m/z

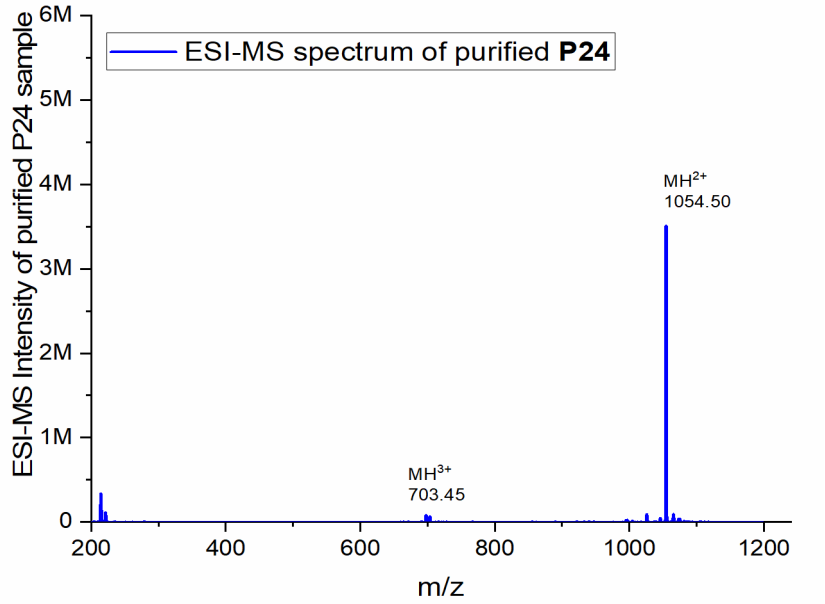

P20
TFA trial cleavage
51%
3b linker modified peptide
49%
immobilisation supernatant
P20
after purification
81%
absorption at 210 nm
time / min

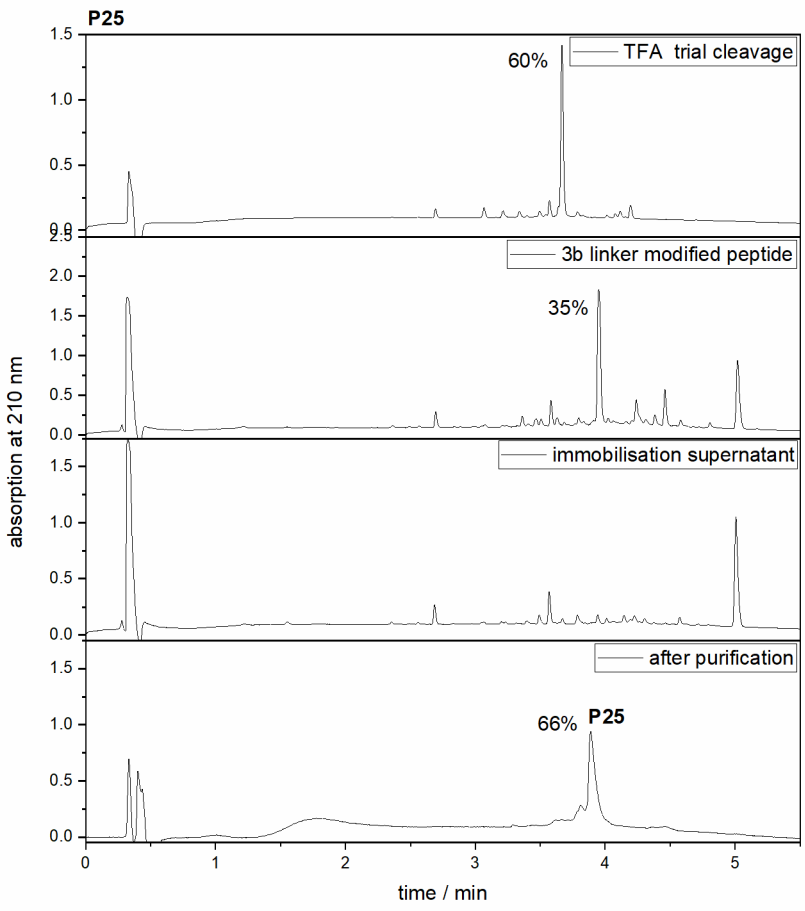

ESI-MS spectrum of purified P20
MH2+
1038.96
MH3+
693.12
ESI-MS Intensity of purified P20 sample
m/z

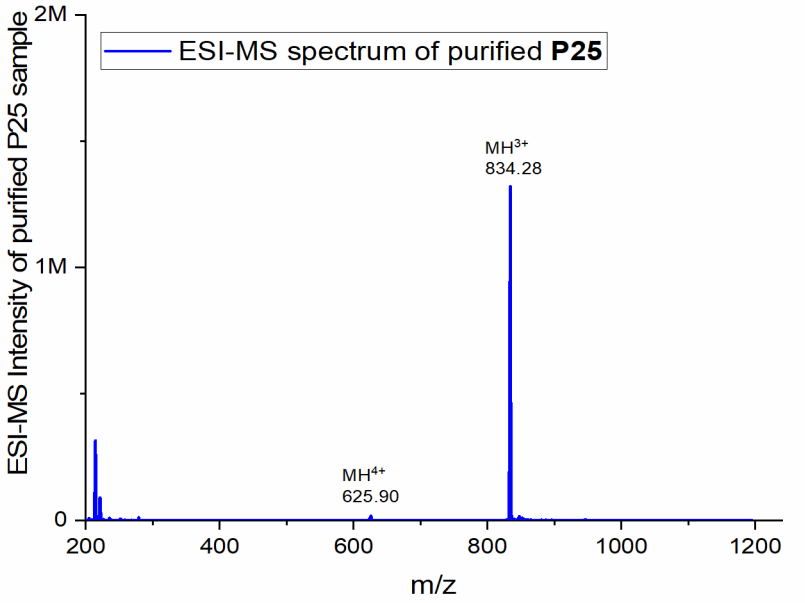

P21
TFA trial cleavage
71%
3b linker modified peptide
45%
immobilisation supernatant
after purification
99%
P21
absorption at 210 nm
time / min
ESI-MS spectrum of purified P21
MH2+
1115.66
MH3+
744.26
ESI-MS Intensity of purified P21 sample
m/z


P23
TFA trial cleavage
85%
3b linker modified peptide
71%
immobilisation supernatant
after purification
P23
90%
absorption at 210 nm
time / min
ESI-MS spectrum of purified P23
MH+
1068.56
MH2+
534.88
ESI-MS Intensity of purified P23 sample
m/z


P24
TFA trial cleavage
30%
3b linker modified peptide
43%
immobilisation supernatant
after purification
P24
75%
absorption at 210 nm
time / min


ESI-MS spectrum of purified P24
MH2+
1054.50
MH3+
703.45
ESI-MS Intensity of purified P24 sample
m/z


P25
TFA trial cleavage
60%
3b linker modified peptide
35%
immobilisation supernatant
after purification
66% P25
absorption at 210 nm
time / min


ESI-MS spectrum of purified P25
MH3+
834.28
MH4+
625.90
ESI-MS Intensity of purified P25 sample
m/z