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Synthesis and SAR of a library of cell-permeable ERY* peptidomimetics inhibiting $\alpha_4\beta_7$ integrin mediated adhesion of TK-1 cells to MAdCAM-1-Fc

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Supplementary Material

General information

All reagents were purchased as reagent grade and used as supplied. Solvents were used as supplied or dried according to standard methods.[1] RP-HPLC solvents were purchased as HPLC grade and used without further purification.

Analytical thin layer chromatography was performed on 0.2 mm aluminium plates of silica gel 60 F_{254} (Merck) and compounds were visualised by ultra-violet fluorescence. Flash chromatography was performed using Davisil® chromatographic silica (LC60Å 40-63 micron) (Grace GmbH & Co.KG) with indicated solvents. Infrared spectra were obtained using a Perkin Elmer Spectrum One Fourier Transform infrared spectrometer with a universal ATR sampling accessory. Nuclear magnetic resonance (NMR) spectra were recorded as indicated on either a Bruker AVANCE DRX300 spectrometer operating on 300 MHz for ^{1}H nuclei and 75 MHz for ^{13}C nuclei or on a Bruker AVANCE DRX400 spectrometer operating on 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei. Chemical shifts are reported in parts per million (ppm) relative to the tetramethylsilane signal at δ_H 0.00 ppm (^{1}H NMR) in $CDCl_3$-$SiMe_4$ solvent or were referenced to the residual methanol signal at δ_H 3.34 ppm in CD_3OD or were referenced to the residual water signal at δ_H 4.79 ppm in D_2O. The ^{13}C values were referenced to the residual chloroform signal at δ_C 77.0 ppm in $CDCl_3$-$SiMe_4$ solvent or residual methanol signal at δ_C 49.15 ppm. ^{1}H NMR data is reported as chemical shift, relative integral, multiplicity (s, singlet; d, doublet; dd, doublet of doublets; dq, doublet of quartets; t, triplet; m, multiplet), coupling constant (J in Hz) and assignment. ^{13}C values are reported as chemical shift (δ_C), degree of hybridisation and assignment. Optical rotations were determined at 20 °C with a Perkin-Elmer 341 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. Melting points were determined on a Electrothermal® melting point apparatus and are uncorrected. Electrospray ionisation mass spectra (ESI-MS) were recorded on a Thermo Finnigan Surveyor MSQ Plus spectrometer or a Bruker micrOTOF-Q II spectrometer. Samples were introduced using direct flow injection at 0.1-0.2 mL/min into an ESI source in positive mode. Major and significant fragments are quoted in the form x (mass to charge ratio). Semi-preparative RP HPLC was performed on a Dionex Ultimate 3000 system using the following column: Phenomenex Gemini $C_{18,}$ 110 Å, 10 mm × 250 mm, 5 μm at a flow rate of 5 mL/min. Analytical RP HPLC was performed on a Dionex P680 system using the following columns: Phenomenex Gemini $C_{18,}$ 110 Å, 4.6 mm × 150 mm, 5 μm (column A) and Phenomenex Gemini $C_{18,}$ 110 Å, 4.6 mm × 250 mm, 5 μm (column B) at a flow rate of 1 mL/min. A linear gradient of 0.1% trifluoroacetic acid-water (A) and 0.1% trifluoroacteic

acid-methanol (B) was used with detection at 210 nm. Gradient systems were adjusted according to the elution profiles and peak profiles obtained from the analytical RP HPLC chromatograms.

Reagents

O-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt) and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were purchased from Advanced ChemTech. *N,N*-Dimethylformamide (DMF) (synthesis grade) was purchased from Scharlau and methanol (HPLC grade) from Merck KGaA. L-3,4-Dihydroxyphenylalanine**,** diisopropylethylamine (DIPEA), piperidine, piperazine, 3,6-dioxa-1,8-octanedithiol, triisopropylsilane (TIS), 4-(dimethylamino)-pyridine (DMAP), 4-(hydroxymethyl)phenylacetic acid (PAM linker) and 4-methylmorpholine (NMM) were purchased from Aldrich. D-biotin, *N,N'*-diisopropylcarbodiimide (DIC), O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) and 4-(hydroxymethyl)phenoxyacetic acid (HMPA) were purchased from GL Biochem. Trifluoroacetic acid (TFA) was purchased from Halocarbon, *p*-cresol from Arcros Organics, $CuSO_4 \cdot 5\ H_2O$ from Ajax Finechem, HF gas from Matheson Tri-Gas and copoly(styrene-1%-divinylbenzene) resin (Bio beads S-X1) 200-400 mesh from Bio-Rad.

All used amino acids were L-amino acids. Fmoc-amino acids were purchased from either CEM corp. or GL Biochem with the following side chain protection: Fmoc-Arg(Pbf)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Phe-OH and Fmoc-Lys(Boc)-OH. Fmoc-L-Tyr(OtBu)-$OCH_2PhOCH_2CH_2CO_2H$ was purchased from PolyPeptide Group.

The commercially available tyrosine analogues Fmoc-L-4-methylphenylalanine, Fmoc-L-4-chlorophenylalanine, Fmoc-L-4-iodophenylalanine, Fmoc-L-4-trifluoromethylphenylalanine, Fmoc-L-2-naphthylalanine, Fmoc-L-4,4'-biphenylalanine, Fmoc-L-homophenylalanine, Fmoc-L-4-nitrophenylalanine and Fmoc-L-4-fluorophenylalanine were purchased from PepTech Corporation and Fmoc-L-3-methylphenylalanine, Fmoc-L-4-cyanophenylalanine, Fmoc-L-4-bromophenylalanine, Fmoc-L-3,4-dichlorophenylalanine, Fmoc-L-3-nitrophenylalanine, Fmoc-L-3,3-diphenylalanine, Fmoc-L-1-naphthylalanine, Fmoc-L-tetrahydroisoquinoline-3-COOH, Fmoc-2-indanylglycine and Fmoc-2-thienylalanine were purchased from Synthetech. Fmoc-L-3-iodophenylalanine was purchased from Chem-Impex International.

Boc-amino acids were purchased from Chem-Impex International or PolyPeptide Group with the following side chain protection: Boc-Asp(O-2-Ada)-OH, Boc-L-Arg(Tos)-OH, Boc-Glu(OcHex)-OH, Boc-Tyr-OH and Boc-L-Tyr(2-Br-Z)-$OCH_2PhCH_2CO_2H$.

Peptide syntheses

Fmoc SPPS

Solid phase peptide syntheses based on Fmoc protection strategy were performed on a 0.1 mmol scale using aminomethylated polystyrene resin[2] (1.0 mmol/g) derivatised with an HMP linker (4-(hydroxymethyl)phenoxyacetic acid, HMPA), the attachment of which is described in **method 1**. Coupling of the first residue was performed according to **method 2** and the level of first residue attachment was estimated using the UV method.[3] For synthesis of the lead peptide carrying non-derivatised tyrosine as C-terminus Fmoc-L-Tyr(OtBu)-$OCH_2PhOCH_2CH_2CO_2H$ was coupled to aminomethylated resin according to **method 3**.

The peptide chains were then assembled using either manual Fmoc SSPS (**method 4**), a Liberty microwave peptide synthesiser (CEM corp.) (**method 5**) or a Tribute^{TM} peptide synthesiser (**method 6**). The peptides were manually capped with D-biotin according to **method 7** and cleaved from resin according to **method 8** or **9**.

Boc SPPS

Solid phase peptide syntheses based on Boc protection strategy were performed on a 0.1 mmol scale using aminomethylated polystyrene resin (1.0 mmol/g) derivatised with PAM linker 4-(hydroxymethyl)phenylacetic acid). The attachment of the linker was carried out according to **method 1** and coupling of the first residue was performed according to **method 2**. For synthesis of the peptide carrying non-derivatised tyrosine as C-terminus Boc-L-Tyr(2-Br-Z)-$OCH_2PhCH_2CO_2H$ was coupled to aminomethylated resin according to **method 3**.

The peptide chains were then assembled using manual *in situ* neutralisation Boc SSPS[4] according to **method 10**, capped with D-biotin according to **method 7** and cleaved from resin according to **method 11**.[5]

General methods

1: Attachment of HMP or PAM linker to aminomethylated PS resin

Aminomethylated resin (0.1 mmol) was swollen in DCM/DMF (1:1) for 20 min and then the solvents were drained. HMPA (3 eq) *or* PAM (3eq) and HOBt (3.8 eq) were dissolved in 3 mL of 20% DMF in DCM and DIC (3 eq) was added. The reaction mixture was added to the resin and agitated for 2 h. The mixture was drained and the resin was washed with DMF (3 ×) and DCM (3 ×).

2: First residue attachment

The Fmoc or Boc protected C-terminal amino acid (4 eq) was dissolved in 3 mL of 20% DMF in DCM. DIC (5 eq) was added and the reaction mixture was added to HMP-resin or PAM-resin. DMAP (1/10 eq in 122 μL of DMF) was added and the mixture agitated for 1.5 h. The mixture was drained and the resin was washed with DMF (3 ×) and DCM (3 ×).

3: Attachment of Fmoc-L-Tyr(OtBu)-$OCH_2PhOCH_2CH_2CO_2H$ or Boc-L-Tyr(2-Br-Z)-$OCH_2PhCH_2CO_2H$ to resin

Aminomethylated resin was swollen in DCM (5 mL) for 15 min and then the solvent was drained. Fmoc-L-Tyr(OtBu)-$OCH_2PhOCH_2CH_2CO_2H$ (2 eq) *or* Boc-L-Tyr(2-Br-Z)-$OCH_2PhCH_2CO_2H$ (2 eq) was dissolved in 1 mL of DCM, DIC (2 eq) was added and the reaction mixture was added to resin followed by agitating for 1h. The mixture was drained and the resin was washed with DMF (3 ×) and DCM (3 ×).

4: Manual Fmoc SPPS

N^{α}-Protected amino acids Fmoc-Glu and Fmoc-Arg (4 eq) were dissolved in 1.5 mL of DMF, HBTU (3.9 eq) was added and shaken until dissolved. Then NMM (8 eq) was added and the mixture was transferred to the reaction vessel. The mixture was shaken for 30 min, filtered and washed with DMF (3 ×) and DCM (3 ×). The N^{α}-protecting group was removed by 20% piperidine solution in DMF (4 mL, 10 min), filtered and washed with DMF (3 ×) and DCM (3 ×).

5: Automated microwave-assisted Fmoc SPPS, Liberty peptide synthesiser

Coupling of Fmoc-Glu (5 eq) was carried out over 5 min at 25 W (73 °C) in presence of HBTU (4.5 eq) and DIPEA (10 eq) in DMF.

Coupling of Fmoc-Arg (5 eq) was carried out over 30 min at room temperature (0 W) followed by 5 min at 25 W (72 °C) in presence of HBTU (4.5 eq) and DIPEA (10 eq) in DMF. The final two Fmoc-Arg residues were attached by double couplings: Fmoc-Arg (5 eq) was coupled for 30 min at room temperature (0 W) followed by 5 min at 25 W (72 °C) in presence of HBTU (4.5 eq) and DIPEA (10 eq) in DMF; coupling was repeated for 5 min at 25 W (73 °C). The N^{α}-protecting group was removed by 20% piperidine solution in DMF or 5% piperazine/0.1 M HOBt solution in DMF (7 mL) with an initial deprotection (30 sec, 62 W, 75 °C) and deprotection (3 min, 60 W, 75 °C) programme.

6: Automated Fmoc SPPS, Tribute™ peptide synthesiser

Couplings of Fmoc-Glu and Fmoc-Arg (5 eq) were carried out in 45 min at room temperature in the presence of HBTU (4.6 eq) and NMM (10 eq) in DMF. The N^{α}-protecting group was removed by 20% piperidine solution in DMF (3 mL, 2 × 5 min).

7: Biotin attachment

D-biotin (4 eq), BOP (4 eq) and HOBt (4 eq) were suspended in 3 mL of DMF. After addition of DIPEA (8 eq) the mixture was added to the resin and agitated for 2 h at rt followed by washing with DMF (3 ×) and DCM (3 ×).

8: Cleavage from resin (Fmoc SPPS)

100 μL TIPS, 250 μL H_2O, 250 μL 3,6-dioxa-1,8-octanedithiol and 9.4 mL TFA were added to the resin and the mixture was agitated for 2 h at room temperature. The TFA solution was filtered and the peptide was precipitated by addition of hexane/diethyl ether (1:1). After centrifugation and washing with hexane/diethyl ether (1:1) the crude peptides were lyophilised from 0.1% trifluoroacetic acid-water.

9: Cleavage from resin for peptides containing TBDMS groups (Fmoc SPPS)[6]

160 μL anisole, 240 μL 1, 2-ethanedithiol, 400 μL thioanisole and finally 7.2 mL TFA were added to the resin and the mixture was agitated for 24 h at room temperature. The TFA solution was filtered and the peptide precipitated by addition of 6 volumes of hexane/diethyl

ether (1:1). After centrifugation and washing the pellet with hexane/diethyl ether (1:1) the crude peptides were lyophilised from 0.1% trifluoroacetic acid-water.

10: Manual *in situ* neutralisation Boc SSPS

Boc-protected amino acids (5 eq) were dissolved in a 0.475 M solution of HBTU in DMF (1 mL), DIPEA (12 eq) was added and the mixture was pre-activated for 1 min. The solution was then transferred to the resin and allowed to react for 15 min. After filtering, the resin was washed with DMF (3 ×) and then treated with neat TFA (10 mL) for 2 min and again washed with DMF (3 ×).

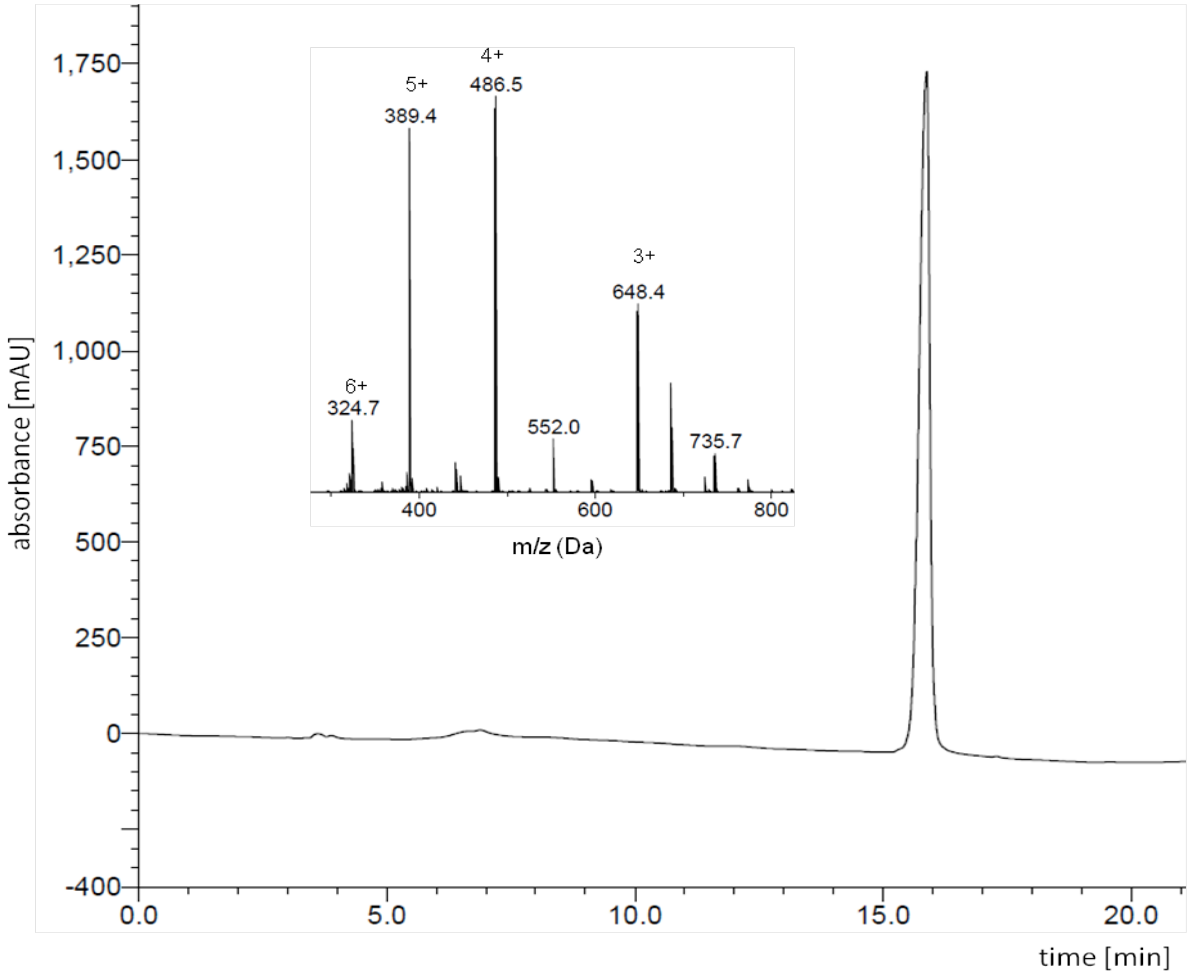
11: Cleavage from resin (Boc SPPS)

The peptides were cleaved from resin by treatment with 10 mL anhydrous HF containing 5 % (v/v) *p*-cresol for 1.5 h at 0 °C. After evaporation of the HF *in vacuo* the crude products were precipitated with chilled diethyl ether (3 ×), dissolved in 50% aqueous acetonitrile containing 0.1% TFA, filtered and lyophilised.

Peptide Syntheses

biotin-R_8ERY (1)

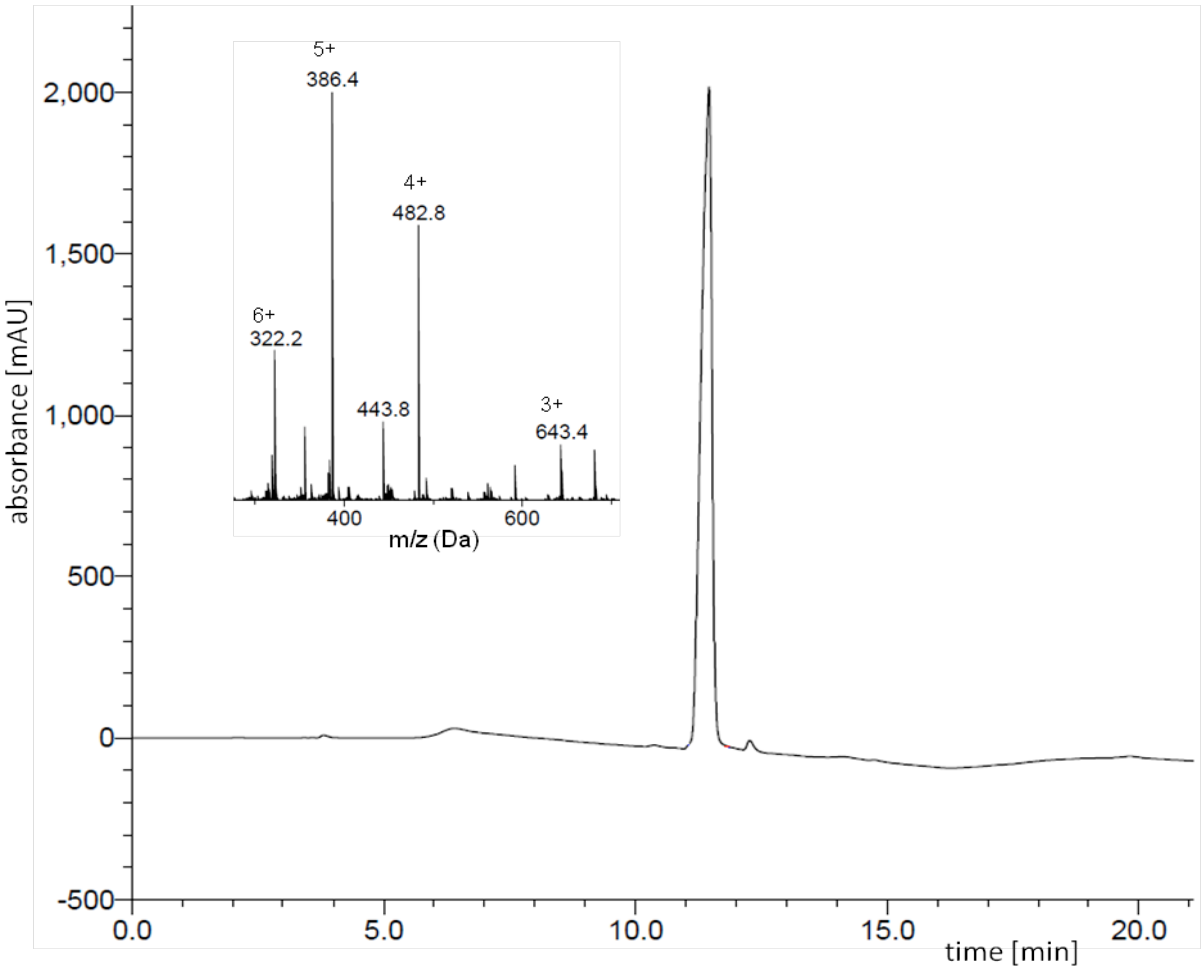
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 242 mg of crude product. The whole amount of crude peptide was purified in a single run by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 17 % B over 16 min followed by a linear gradient of 17% B to 40% B over 230 min. Lyophilisation yielded the purified peptide (76 mg, 39%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 15.9 min (Phenomenex Gemini C_{18}, 1-50% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+6H$^+$] calculated mass = 324.7, observed mass = 324.7; [M+5H$^+$] calculated mass = 389.5, observed mass = 389.4; [M+4H$^+$] calculated mass = 486.6, observed mass = 486.5; [M+3H$^+$] calculated mass = 648.4, observed mass = 648.4.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY including ESI-MS spectrum

biotin-R_8DRY (2)

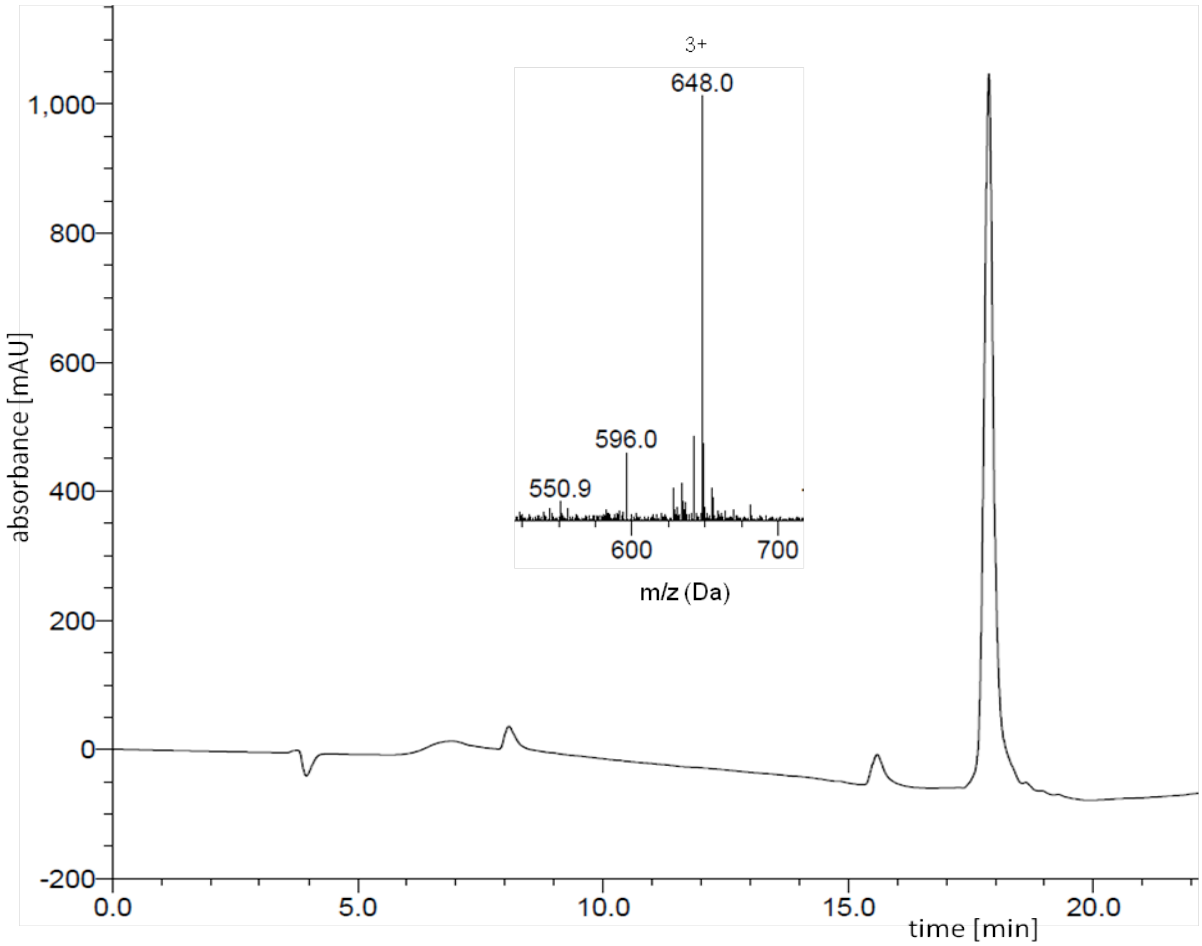
The peptide chain was assembled using Boc SPPS according to **method 10**, capped with biotin for 15 min and then cleaved according to **method 11** yielding 303 mg of crude product. 12 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80% B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 52%) as a white solid in *ca.* 98% purity according to analytical HPLC (column A). R_t 11.5 min (Phenomenex Gemini C_{18}, 1-80% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+6H$^+$] calculated mass = 322.2, observed mass = 322.2; [M+5H$^+$] calculated mass = 386.4, observed mass = 386.4; [M+4H$^+$] calculated mass = 482.8, observed mass = 482.8; [M+3H$^+$] calculated mass = 643.4, observed mass = 643.4.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R8DRY including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-methylphenylalanine) (3)

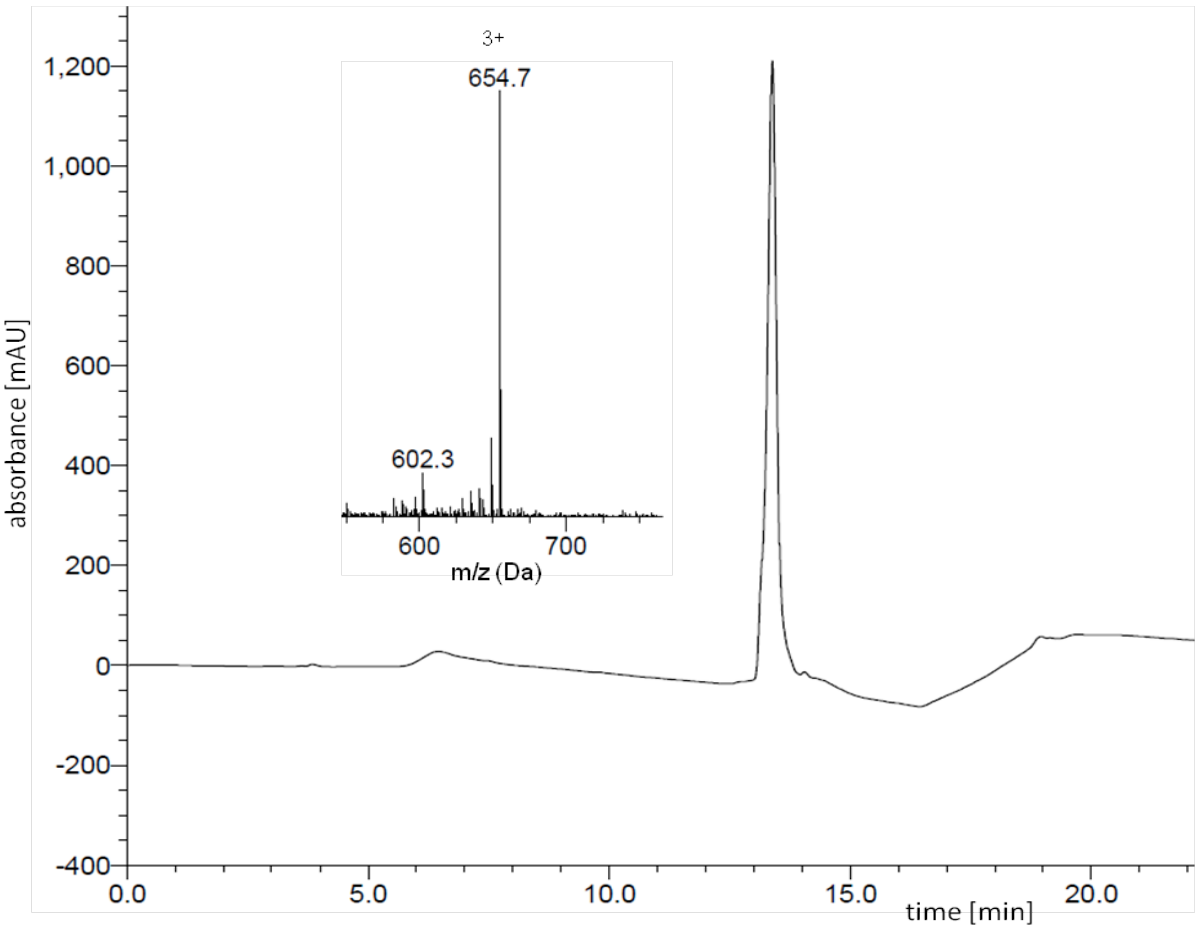
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 114 mg of crude product. 20 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 40 % B over 40 min. Lyophilisation yielded the purified peptide (8 mg, 25%) as a white solid in *ca.* 94% purity according to analytical HPLC (column A). R_t 17.9 min (Phenomenex Gemini C_{18}, 1-50% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 647.8, observed mass = 648.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-methylphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-chlorophenylalanine) (4)

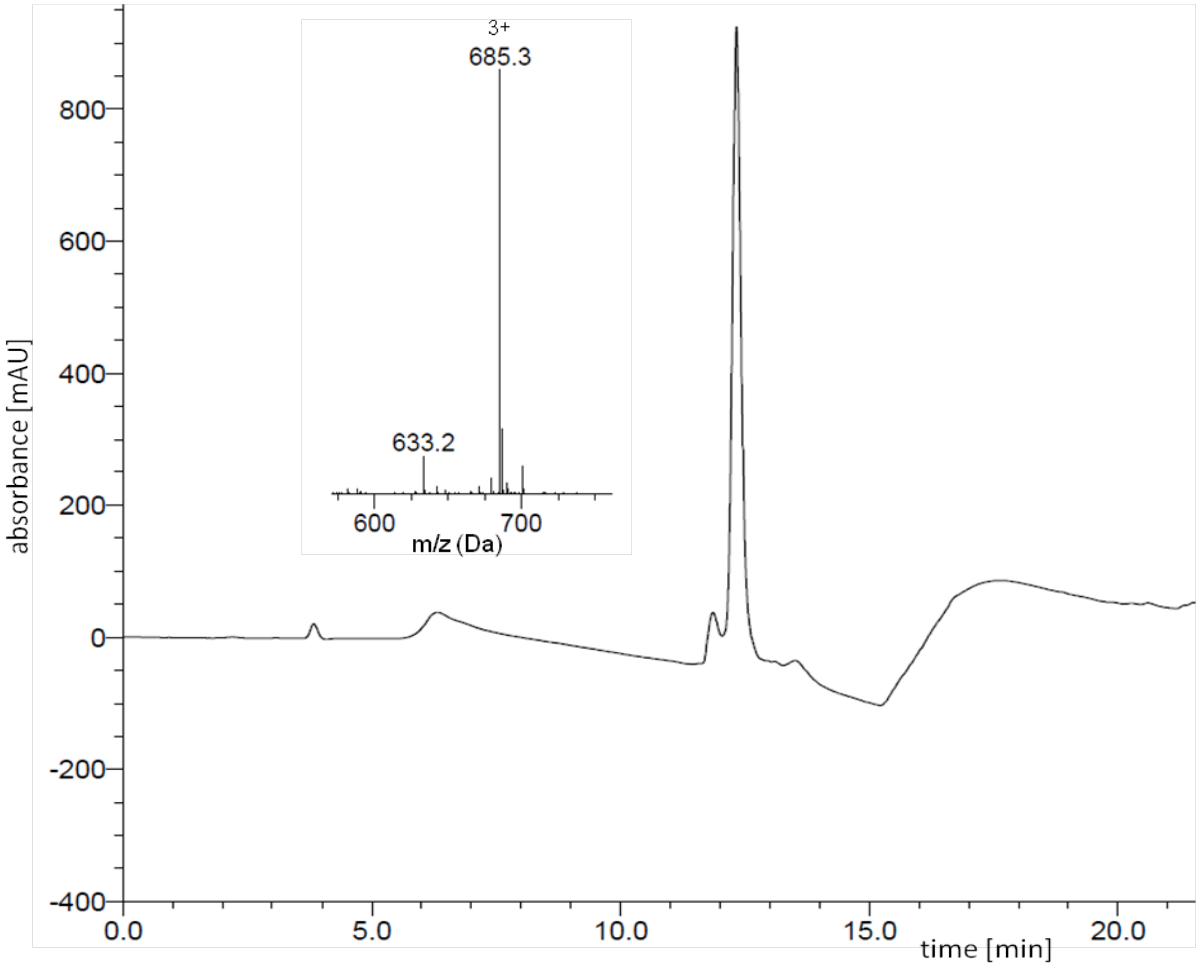
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 46 mg of crude product. 19 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 40 % B over 60 min. Lyophilisation yielded the purified peptide (3 mg, 6%) as a white solid in *ca.* 97% purity according to analytical HPLC (column A). R_t 13.4 min (Phenomenex Gemini C_{18}, 1-80% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 654.6, observed mass = 654.7.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-chlorophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-iodophenylalanine) (5)

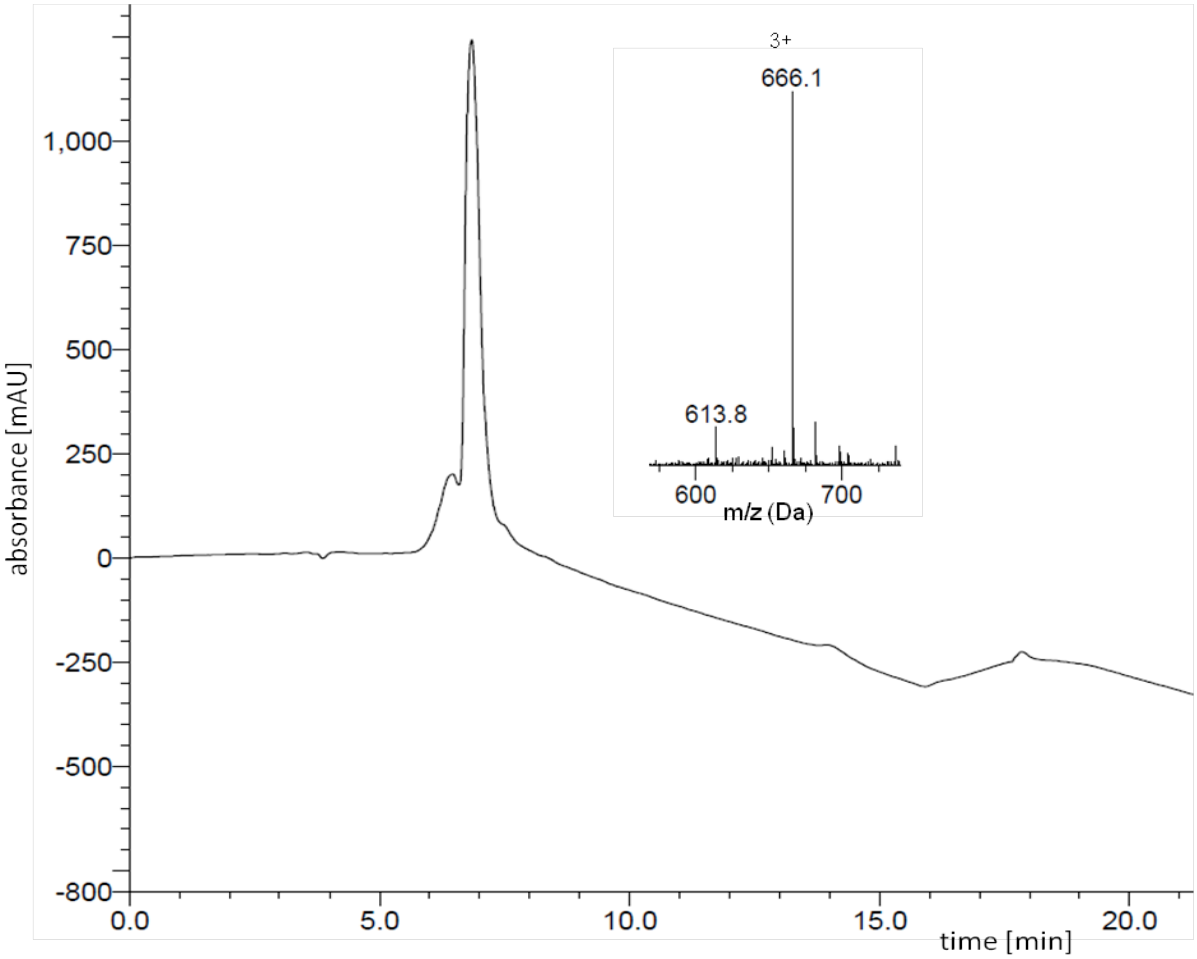
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 91 mg of crude product. 23 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 40 % B over 40 min. Lyophilisation yielded the purified peptide (4 mg, 12%) as a white solid in *ca.* 92% purity according to analytical HPLC (column A). R_t 12.3 min (Phenomenex Gemini C_{18}, 1-100% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 685.1, observed mass = 685.3.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-iodophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-trifluoromethylphenylalanine) (6)

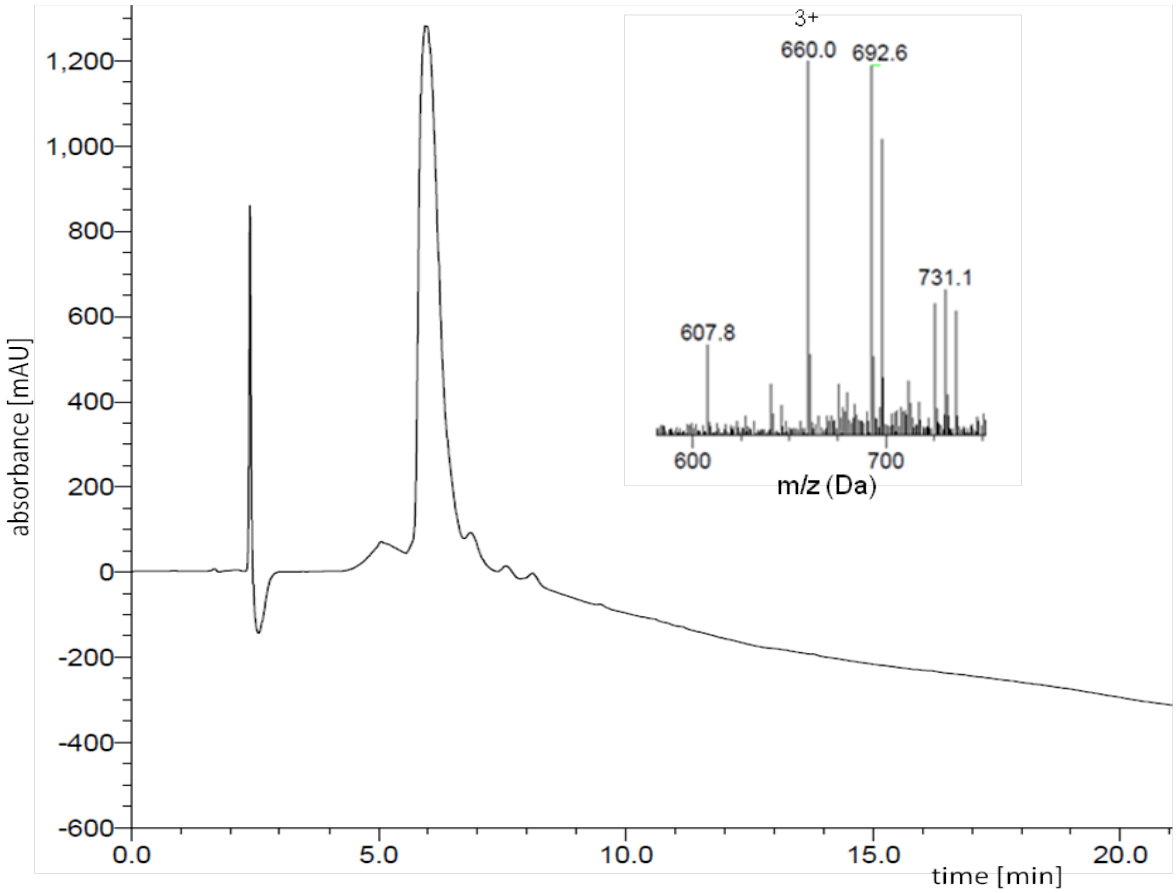
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 216 mg of crude product. 30 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 40 % B over 60 min. Lyophilisation yielded the purified peptide (13 mg, 72%) as a white solid in *ca.* 86% purity according to analytical HPLC (column A). R_t 6.8 min (Phenomenex Gemini C_{18}, 1-90% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 665.8, observed mass = 666.1.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-trifluoromethylphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 2-naphthylalanine) (7)

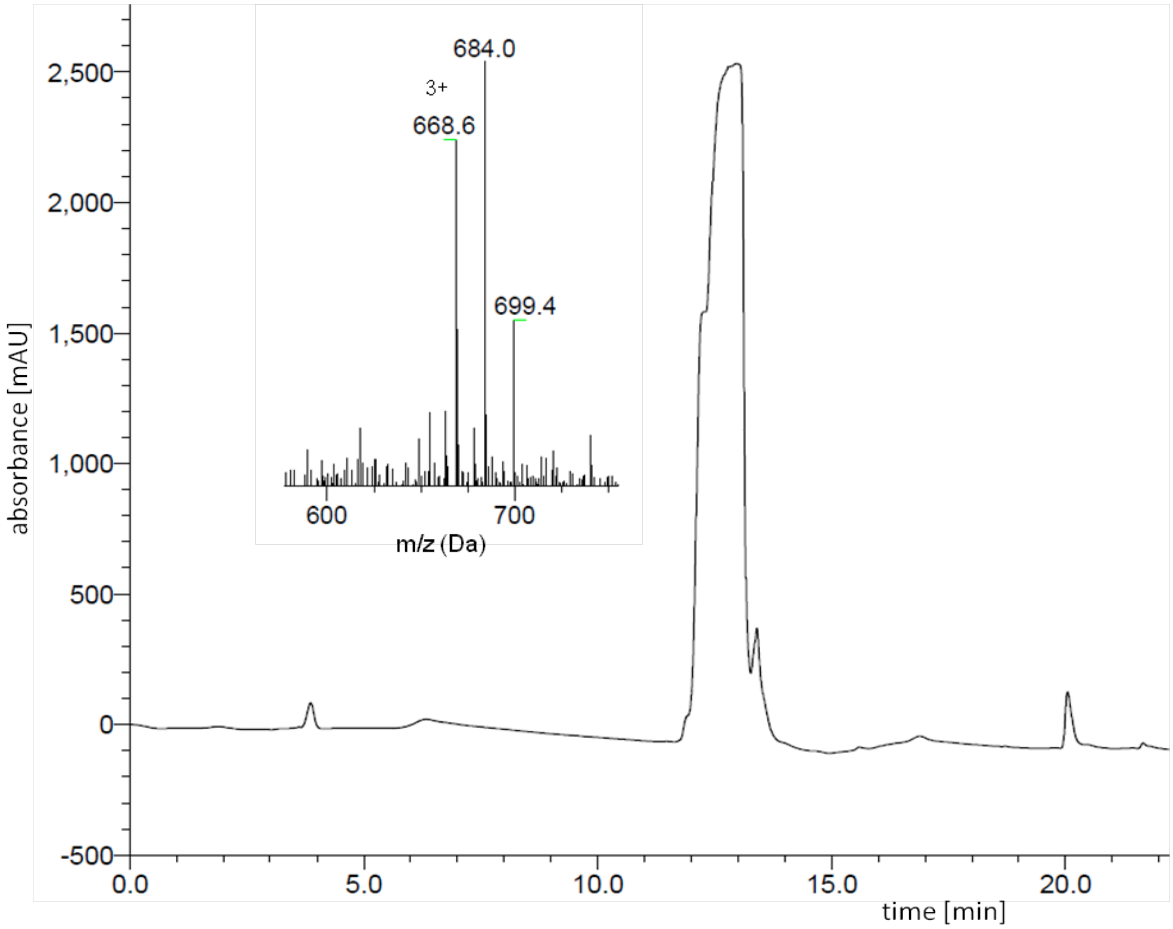
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 116 mg of crude product. 30 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 50 min. Lyophilisation yielded the purified peptide (9 mg, 25%) as a white solid in *ca.* 93% purity according to analytical HPLC (column A). R_t 5.9 min (Phenomenex Gemini C_{18}, 1-70% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 659.8, observed mass = 660.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 2-naphthylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4, 4'-biphenylalanine) (8)

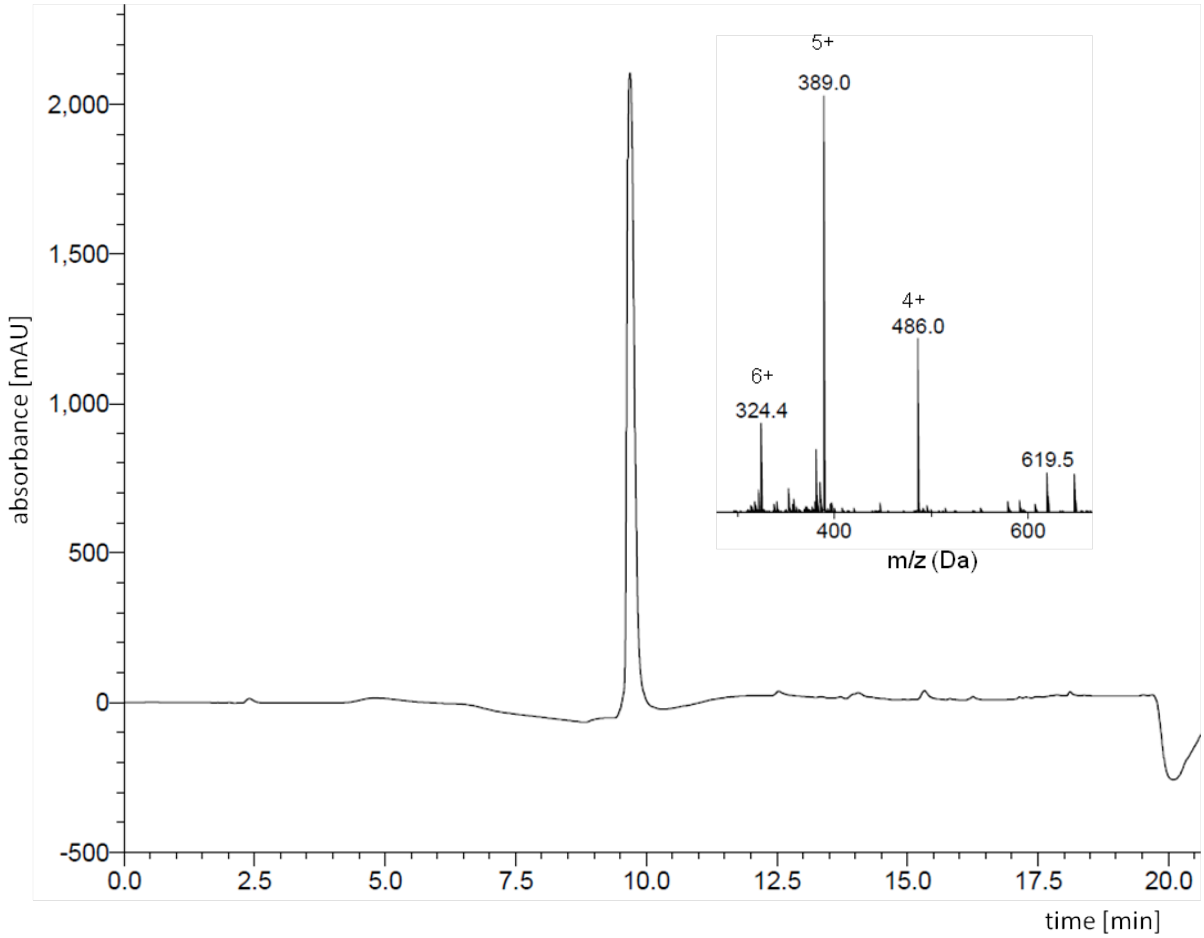
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 234 mg of crude product. 46 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 40 min. Lyophilisation yielded the purified peptide (20 mg, 59%) as a white solid in *ca.* 91% purity according to analytical HPLC (column A). R_t 13.0 min (Phenomenex Gemini C_{18}, 1-100% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+3H$^+$] calculated mass = 668.5, observed mass = 668.6.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4,4'-biphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = homophenylalanine) (9)

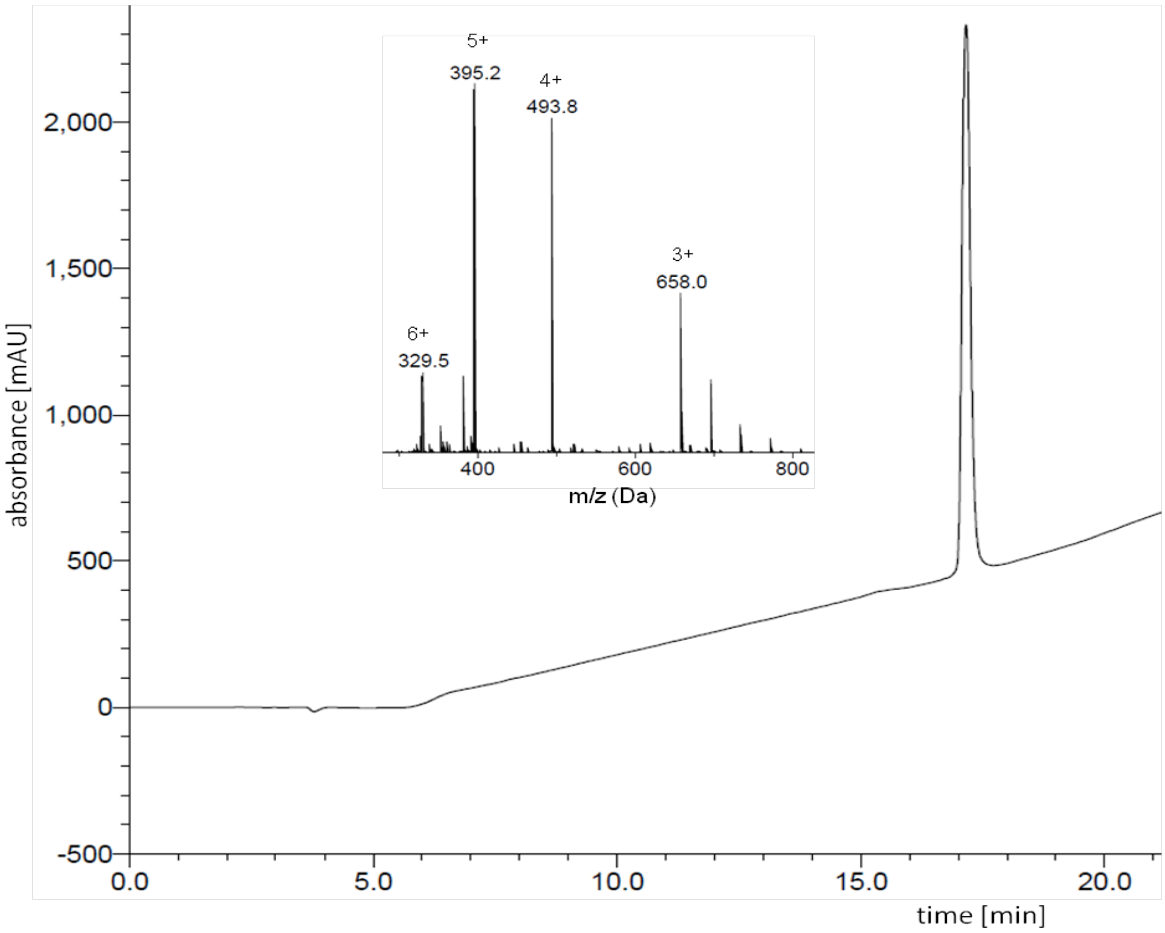
The peptide chain was assembled according to **method 4**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 198 mg of crude product. 9 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 73%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 9.7 min (Phenomenex Gemini C_{18}, 1-100% B over 15 min, 1 mL/min); *m/z* (ESI-MS): [M+6H$^+$] calculated mass = 324.4, observed mass = 324.4; [M+5H$^+$] calculated mass = 389.1, observed mass = 389.0; [M+4H$^+$] calculated mass = 486.1, observed mass = 486.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = homophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-nitrophenylalanine) (10)

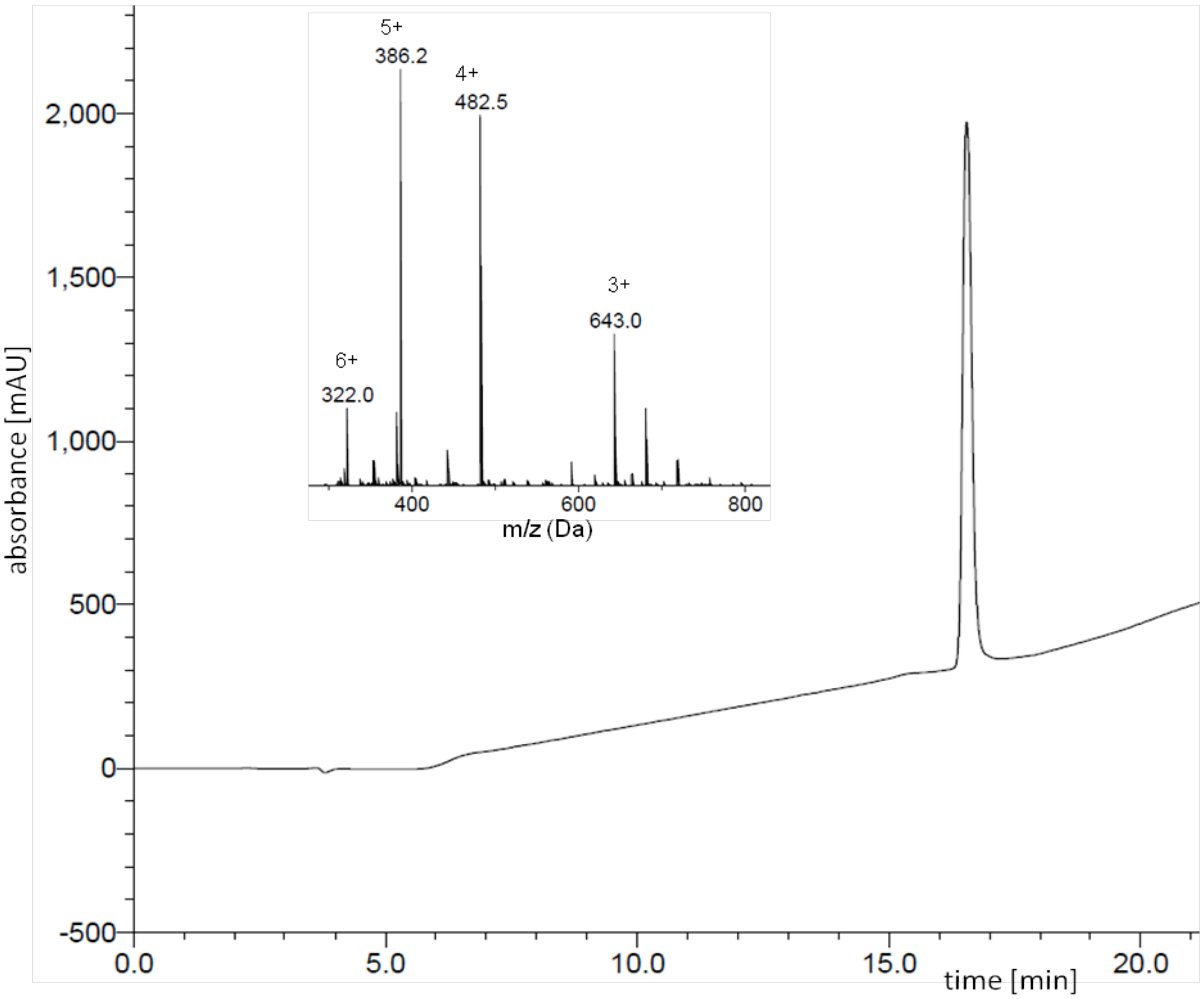
The peptide chain was assembled according to **method 4**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 325 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (6 mg, 92%) as a white solid in *ca.* 99% purity according to analytical HPLC (column B). R_t 17.2 min (Phenomenex Gemini C_{18}, 1-60% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+6H$^+$] calculated mass = 329.5, observed mass = 329.5; [M+5H$^+$] calculated mass = 395.3, observed mass = 395.2; [M+4H$^+$] calculated mass = 493.8, observed mass = 493.8; [M+3H$^+$] calculated mass = 658.1, observed mass = 658.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-nitrophenylalanine) including ESI-MS spectrum

biotin-R_8ERF (11)

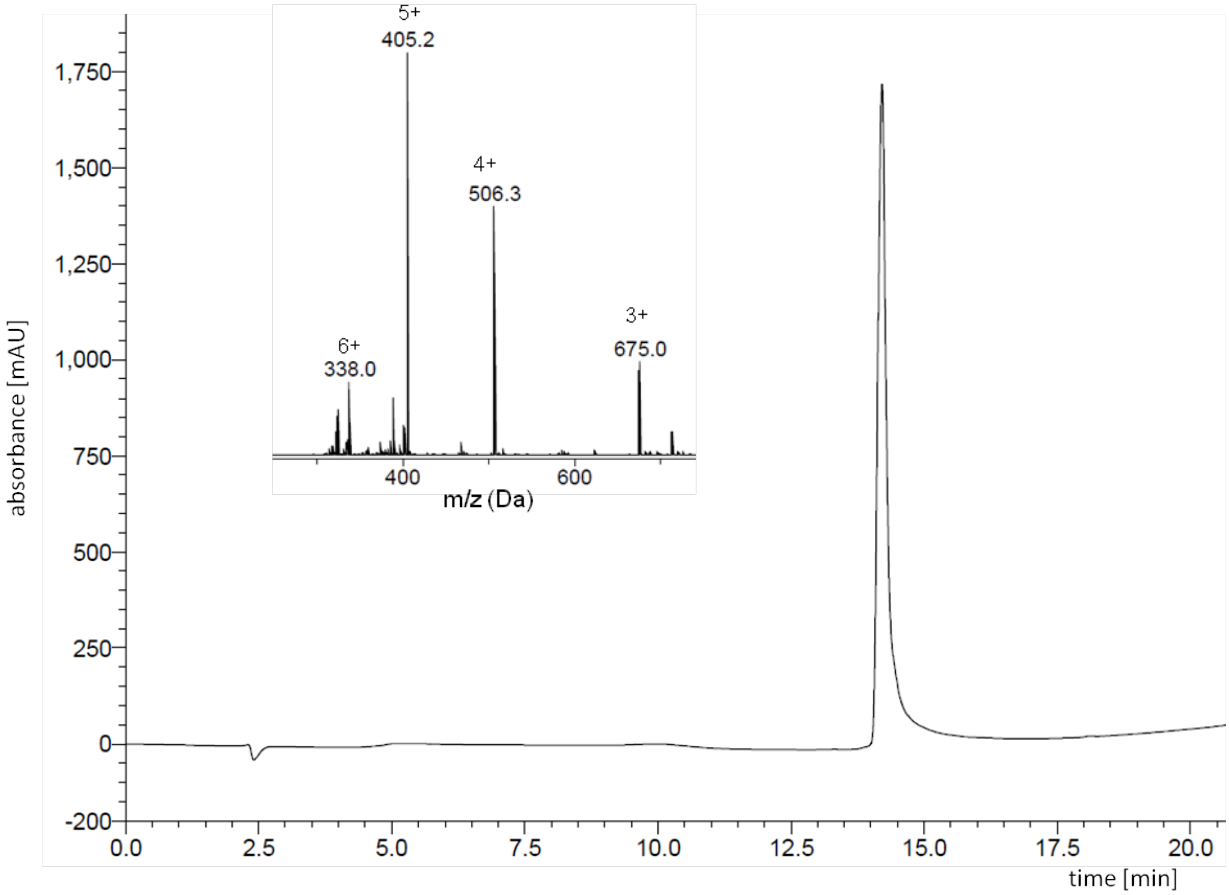
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 142 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (6 mg, 44%) as a white solid in *ca.* 99% purity according to analytical HPLC (column B). R_t 16.5 min (Phenomenex Gemini C_{18}, 1-60% B over 20 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 322.0, observed mass = 322.0; [M+5H$^+$] calculated mass = 386.3, observed mass = 386.2; [M+4H$^+$] calculated mass = 482.6, observed mass = 482.5; [M+3H$^+$] calculated mass = 643.1, observed mass = 643.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERF including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-sulfotyrosine) (12)

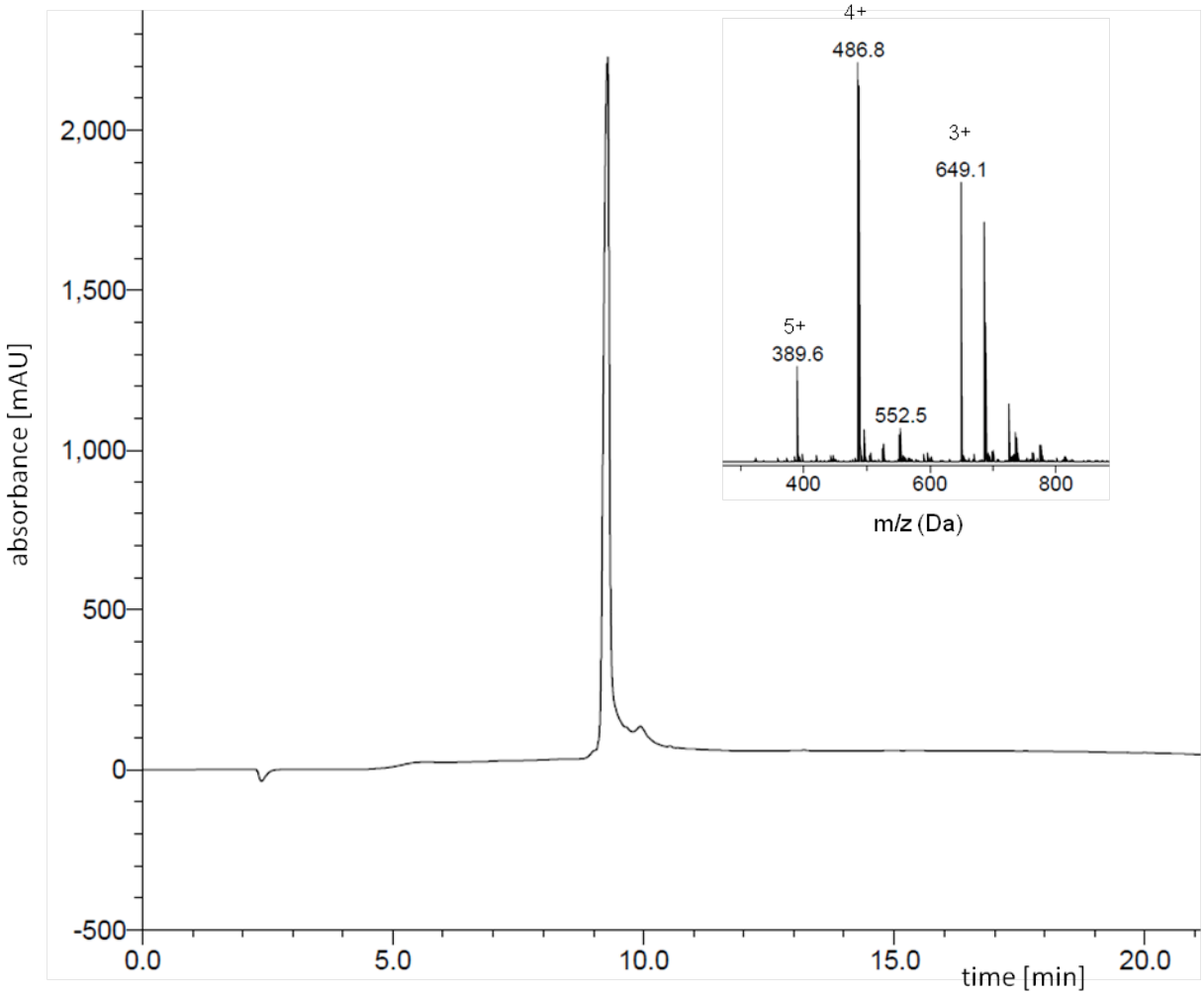
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 174 mg of crude product. 25 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80% B over 40 min. Lyophilisation yielded the purified peptide (3 mg, 29%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 14.2 min (Phenomenex Gemini C_{18}, 1-61% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+6H$^+$] calculated mass = 338.1, observed mass = 338.0; [M+5H$^+$] calculated mass = 405.5, observed mass = 405.2; [M+4H$^+$] calculated mass = 506.6, observed mass = 506.3; [M+3H$^+$] calculated mass = 675.1, observed mass = 675.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-sulfotyrosine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-fluorophenylalanine) (13)

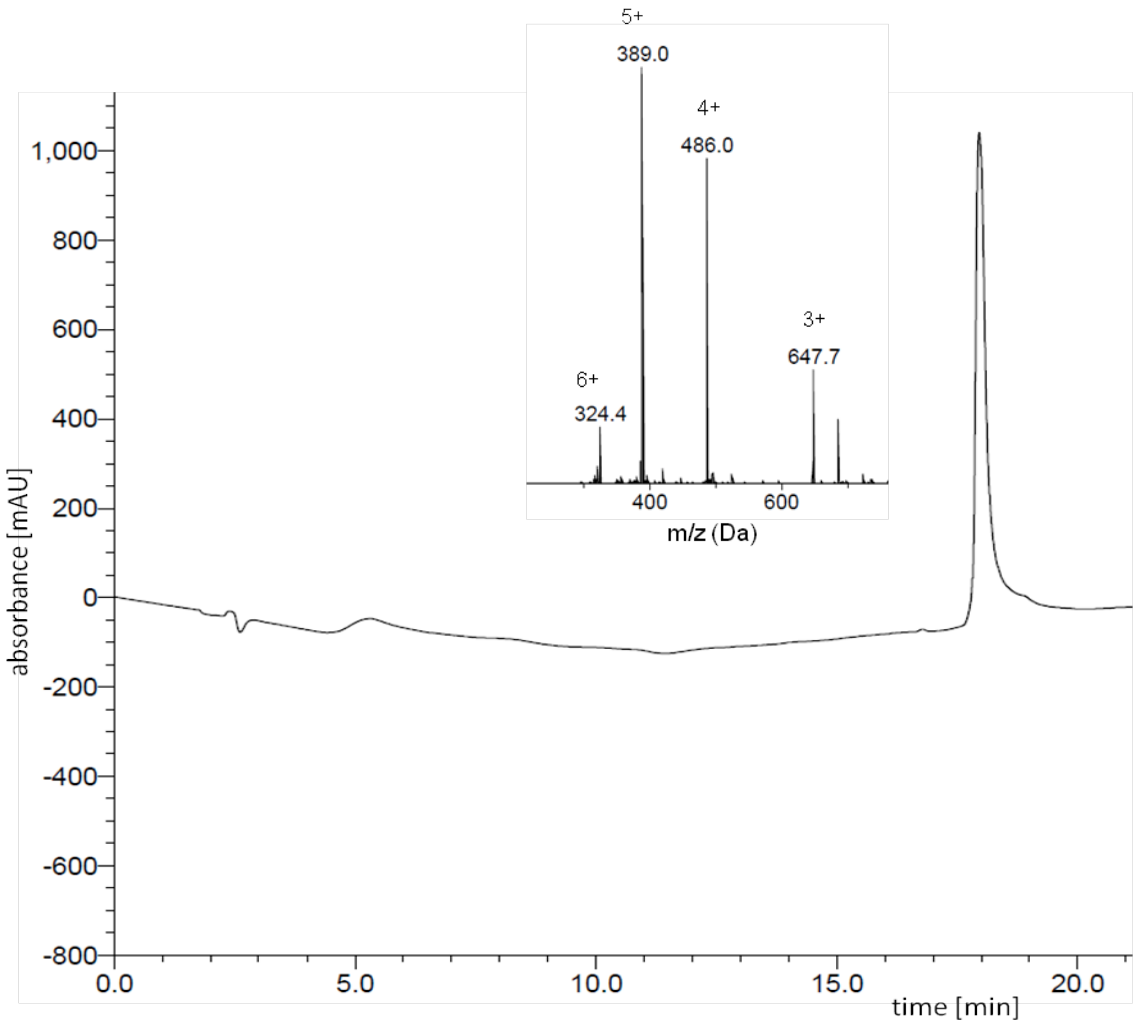
The peptide chain was assembled according to **method 4**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 226 mg of crude product. 24 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 60 % B over 50 min. Lyophilisation yielded the purified peptide (8 mg, 87%) as a white solid in *ca.* 95% purity according to analytical HPLC (column A). R_t 9.3 min (Phenomenex Gemini C_{18}, 1-61% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+5H$^+$] calculated mass = 389.9, observed mass = 389.6; [M+4H$^+$] calculated mass = 487.1, observed mass = 486.8; [M+3H$^+$] calculated mass = 649.1, observed mass = 649.1.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-fluorophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 3-methylphenylalanine) (14)

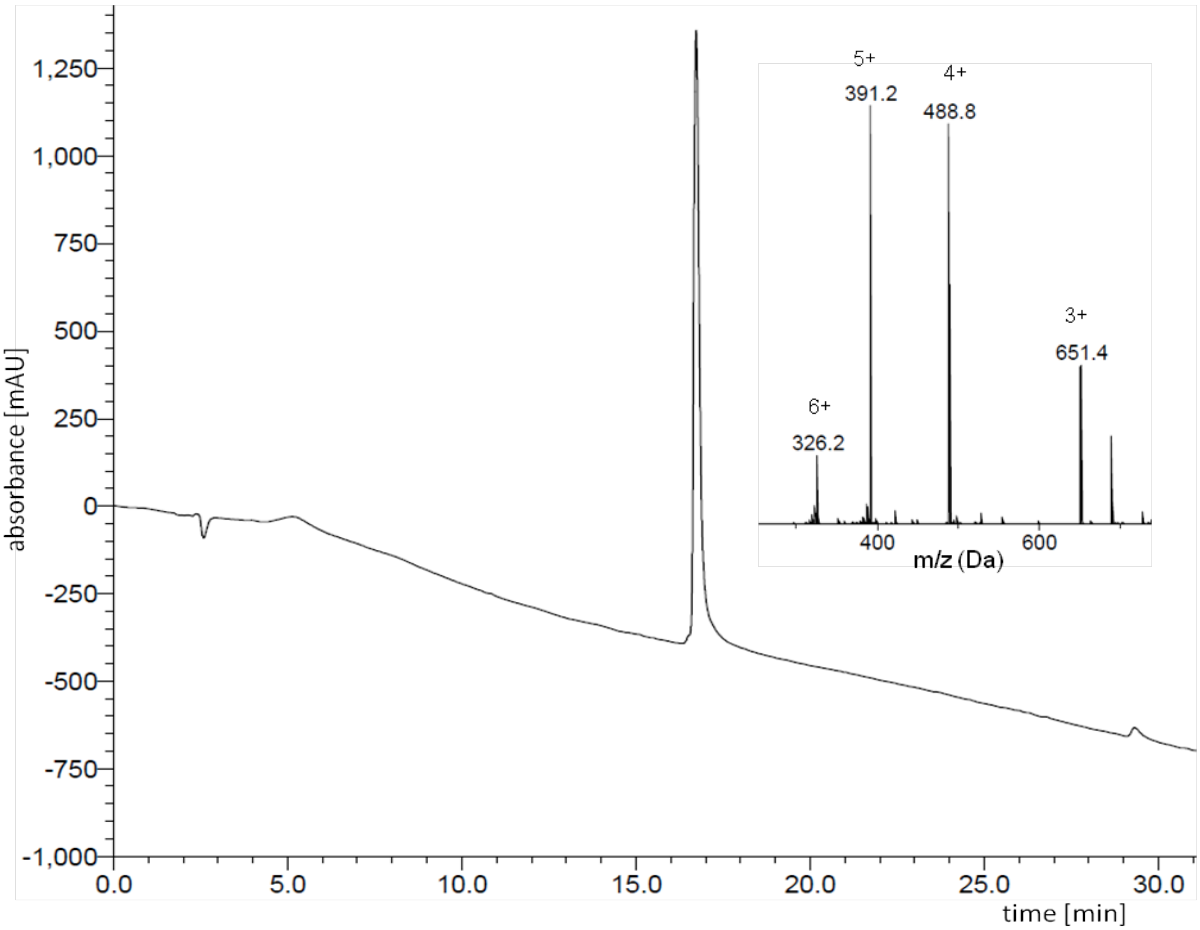
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 159 mg of crude product. 11 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 57%) as a white solid in *ca.* 96% purity according to analytical HPLC (column A). R_t 18.0 min (Phenomenex Gemini C_{18}, 1-60% B over 20 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 324.4, observed mass = 324.4; [M+5H$^+$] calculated mass = 389.1, observed mass = 389.0; [M+4H$^+$] calculated mass = 486.1, observed mass = 486.0; [M+3H$^+$] calculated mass = 647.8, observed mass = 647.7.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 3-methylphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-cyanophenylalanine) (15)

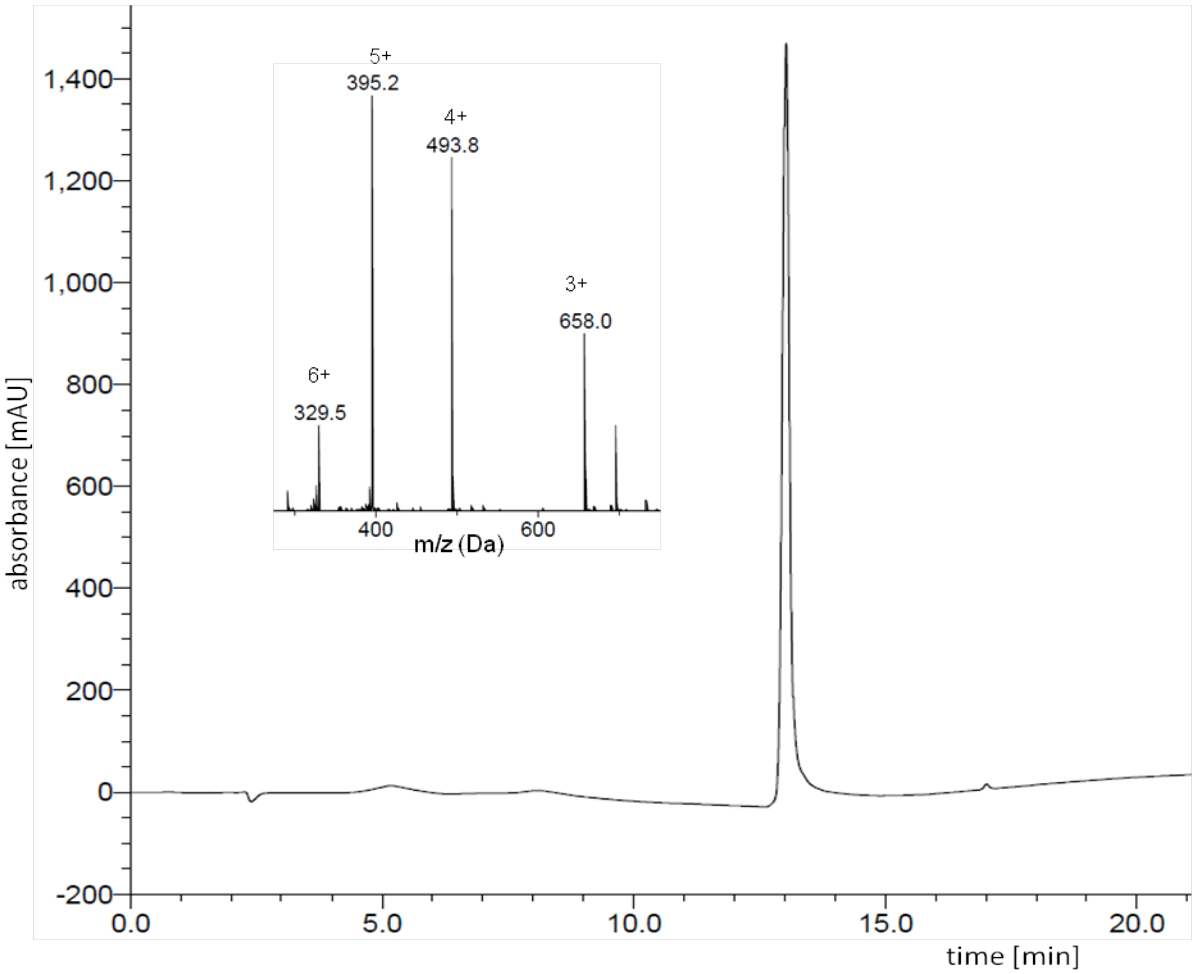
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 170 mg of crude product. 11 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 43%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 16.7 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 326.2, observed mass = 326.2; [M+5H$^+$] calculated mass = 391.3, observed mass = 391.2; [M+4H$^+$] calculated mass = 488.8, observed mass = 488.8; [M+3H$^+$] calculated mass = 651.4, observed mass = 651.4.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-cyanophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 3-nitrophenylalanine) (16)

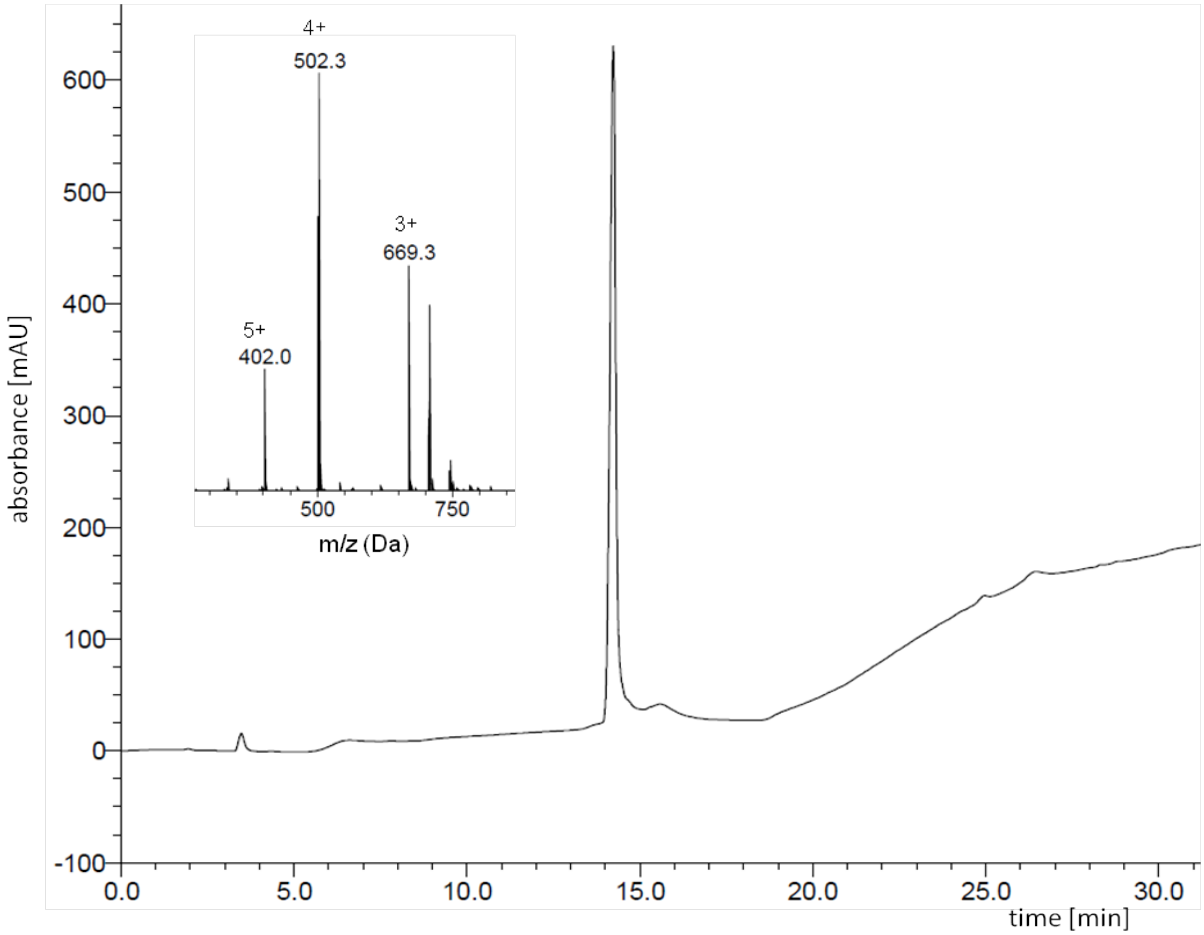
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 195 mg of crude product. 15 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (6 mg, 65%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 13.0 min (Phenomenex Gemini C_{18}, 1-60% B over 20 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 329.5, observed mass = 329.5; [M+5H$^+$] calculated mass = 395.3, observed mass = 395.2; [M+4H$^+$] calculated mass = 493.8, observed mass = 493.8; [M+3H$^+$] calculated mass = 658.1, observed mass = 658.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 3-nitrophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-bromophenylalanine) (17)

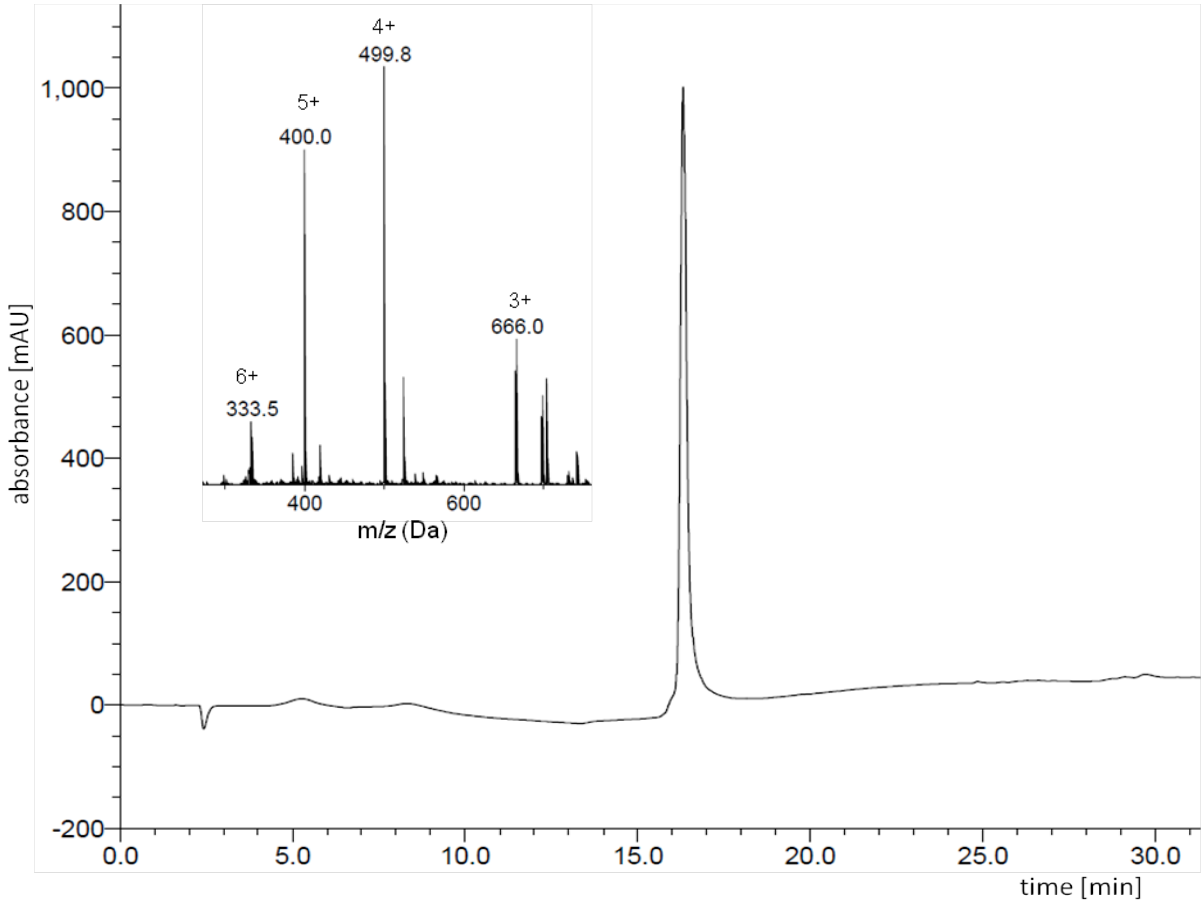
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 180 mg of crude product. 12 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (2 mg, 28%) as a white solid in *ca.* 92% purity according to analytical HPLC (column A). R_t 14.2 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 335.2, observed mass = 335.2; [M+5H$^+$] calculated mass = 402.0, observed mass = 402.0; [M+4H$^+$] calculated mass = 502.3, observed mass = 502.3; [M+3H$^+$] calculated mass = 669.4 , observed mass = 669.3.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-bromophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 3,4-dichlorophenylalanine) (18)

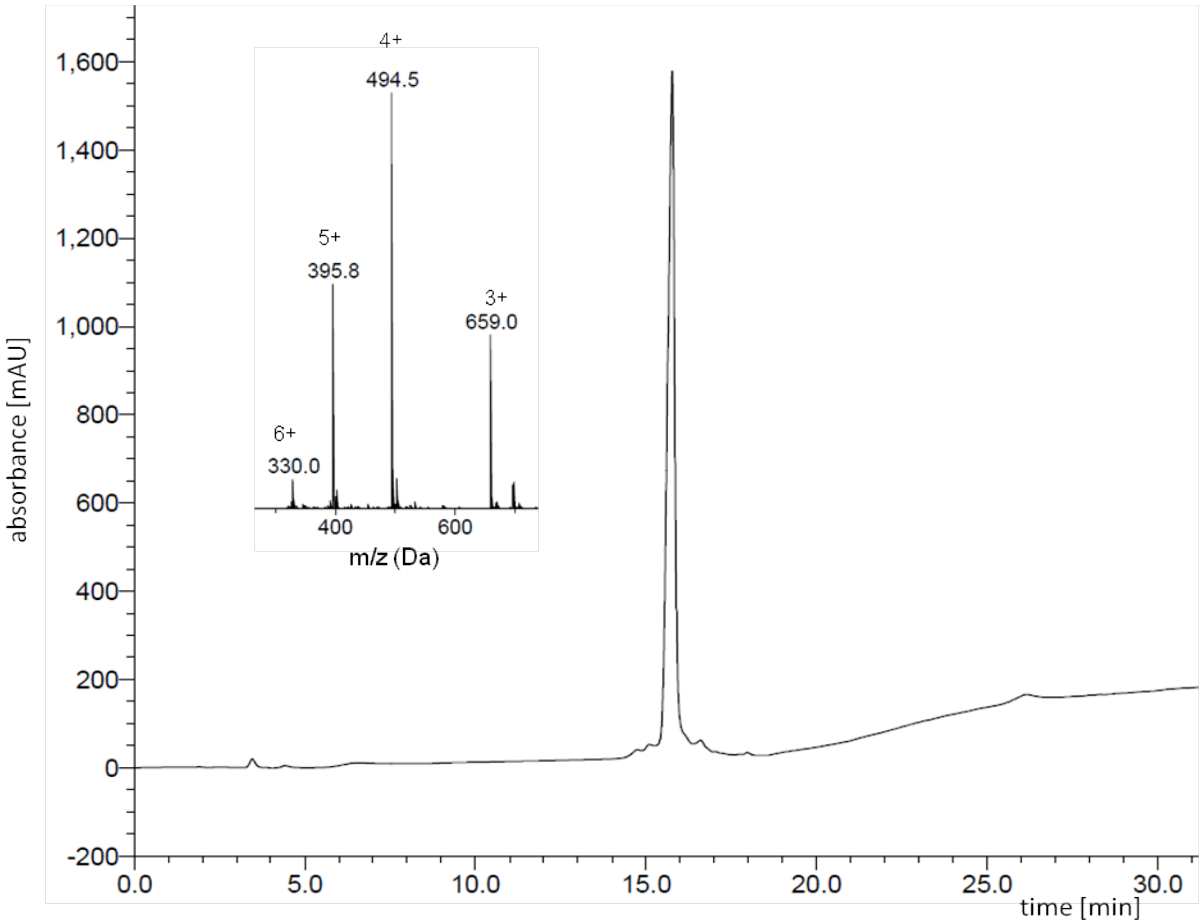
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 174 mg of crude product. 15 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (7 mg, 54%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 16.3 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 333.5, observed mass = 333.5; [M+5H$^+$] calculated mass = 400.0, observed mass = 400.0; [M+4H$^+$] calculated mass = 499.8, observed mass = 499.8; [M+3H$^+$] calculated mass = 666.1, observed mass = 666.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 3,4-dichlorophenylalanine) including ESI-MS spectrum

biotin-R_8EKY* (Y* = 4,4'-biphenylalanine) (19)

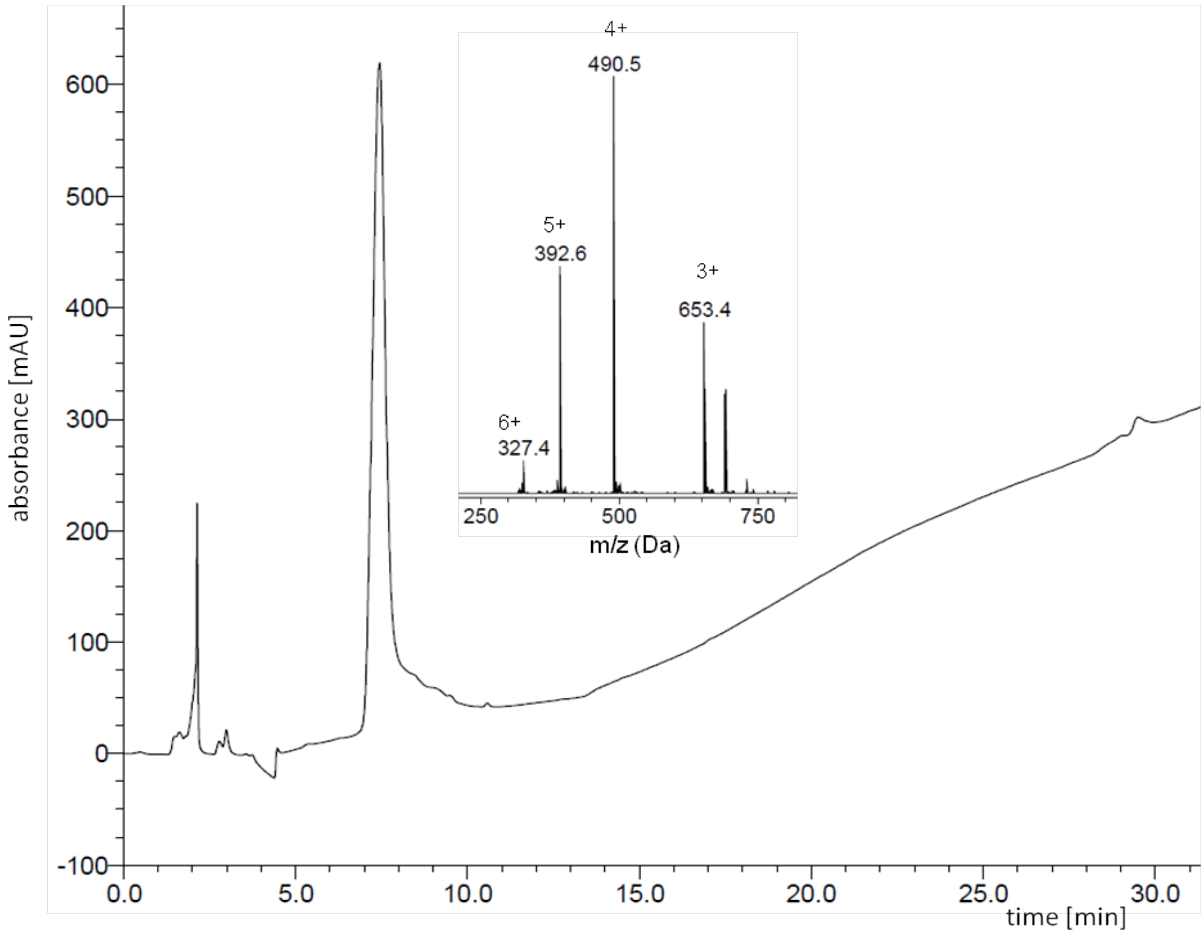
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 145 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (6 mg, 64%) as a white solid in *ca.* 99% purity according to analytical HPLC (column B). R_t 15.8 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 330.1, observed mass = 330.0; [M+5H$^+$] calculated mass = 395.9, observed mass = 395.8; [M+4H$^+$] calculated mass = 494.6, observed mass = 494.5; [M+3H$^+$] calculated mass = 659.1, observed mass = 659.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8EKY* (Y* = 4,4'-biphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 3,4-dihydroxyphenylalanine) (20)

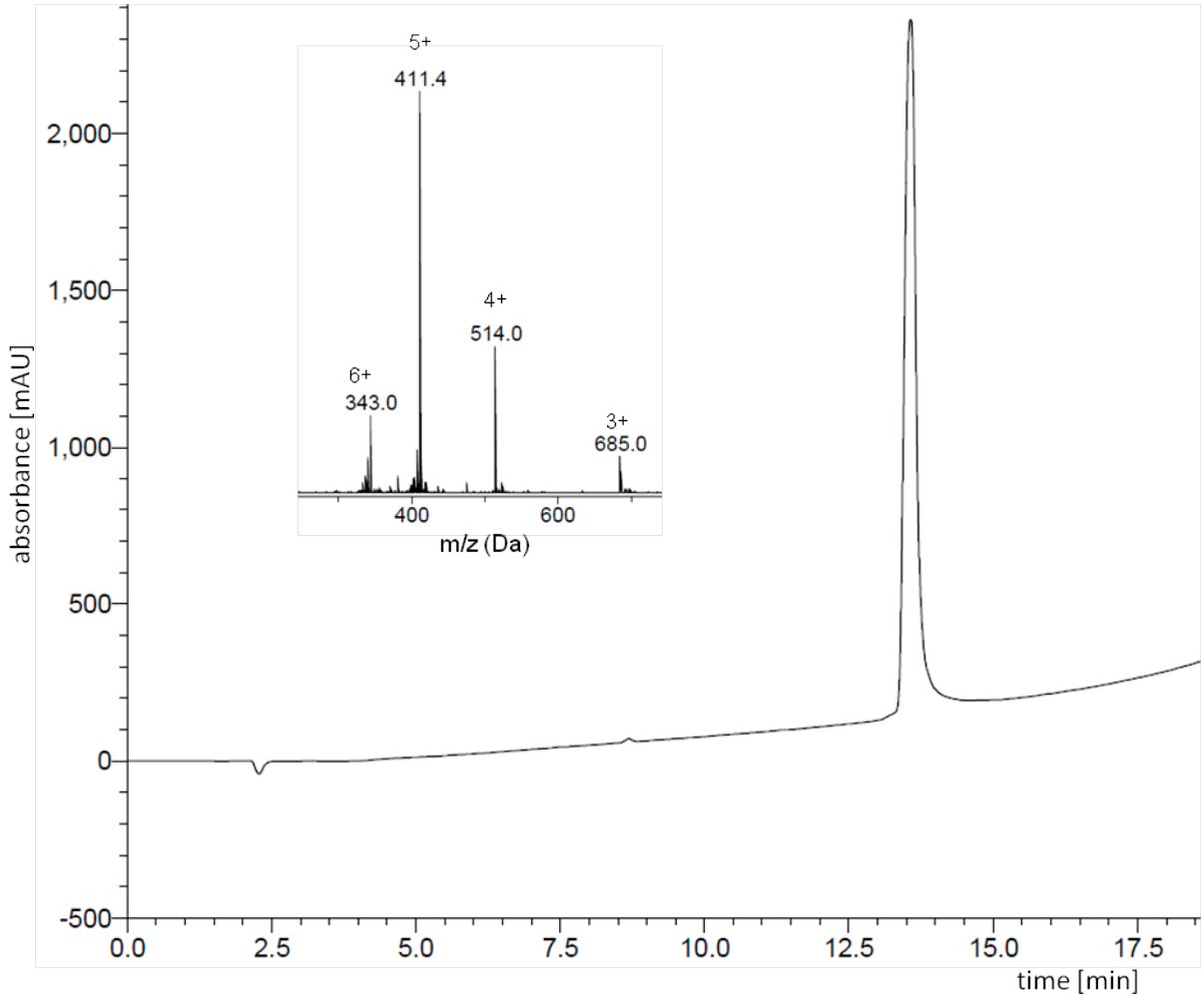
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 9** yielding 182 mg of crude product. 11 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (2 mg, 36%) as a white solid in *ca.* 93% purity according to analytical HPLC (column A). R_t 7.4 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 327.4, observed mass = 327.4; [M+5H$^+$] calculated mass = 392.7, observed mass = 392.6; [M+4H$^+$] calculated mass = 490.6, observed mass = 490.5; [M+3H$^+$] calculated mass = 653.4, observed mass = 653.4.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 3,4-dihydroxyphenylalanine) including ESI-MS spectrum

biotin-R_8ERY^* (Y^* = 3-iodophenylalanine) (21)

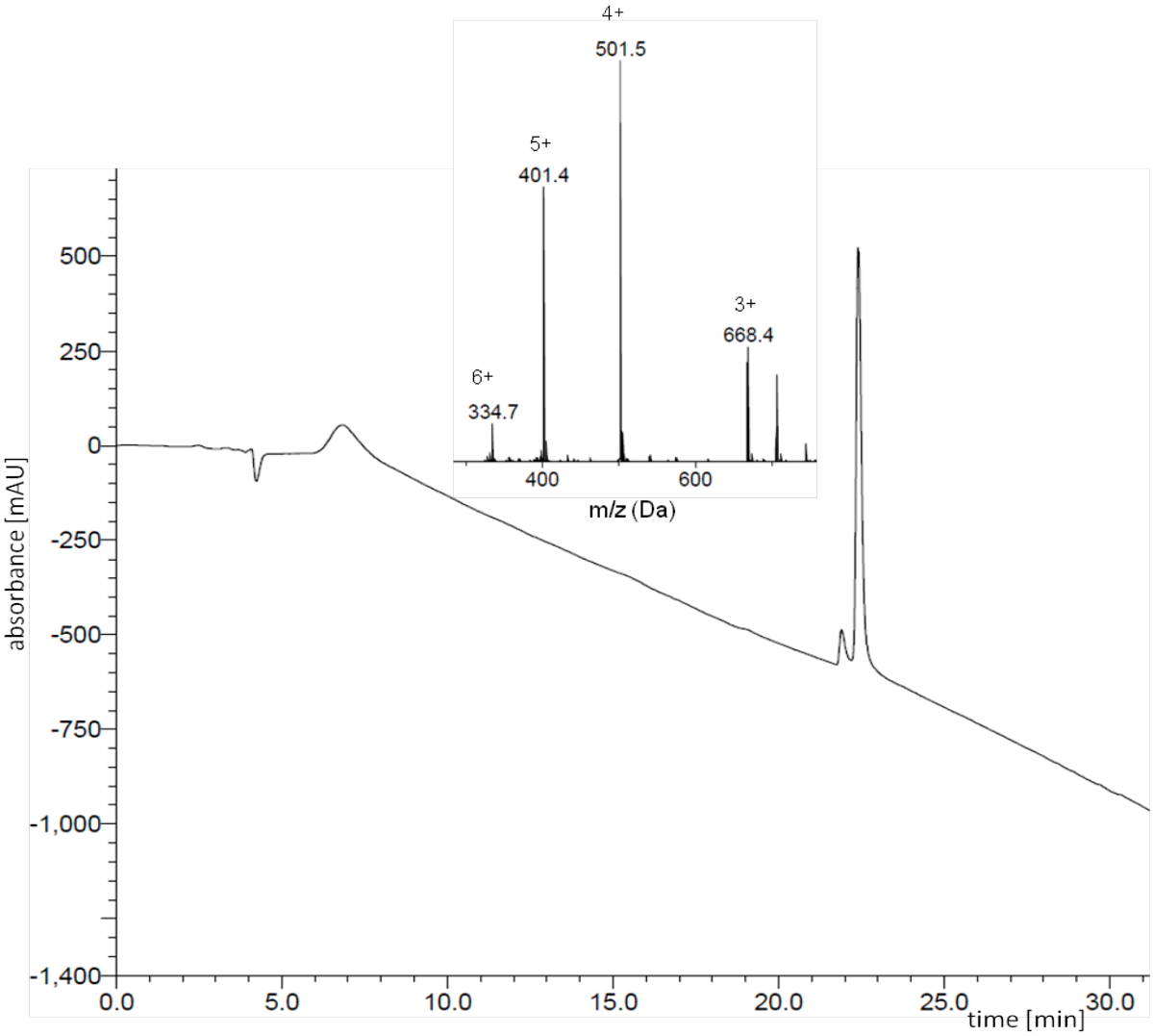
The peptide chain was assembled according to **method 4**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 125 mg of crude product. 20 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 60 % B over 60 min. Lyophilisation yielded the purified peptide (2 mg, 18%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 13.6 min (Phenomenex Gemini C_{18}, 10-60% B over 15 min, 1 mL/min); *m/z* (ESI-MS): $[M+6H^+]$ calculated mass = 343.0, observed mass = 343.0; $[M+5H^+]$ calculated mass = 411.4, observed mass = 411.4; $[M+4H^+]$ calculated mass = 514.1, observed mass = 514.0; $[M+3H^+]$ calculated mass = 685.1, observed mass = 685.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY^* (Y^* = 3-iodophenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 3,3-diphenylalanine) (22)

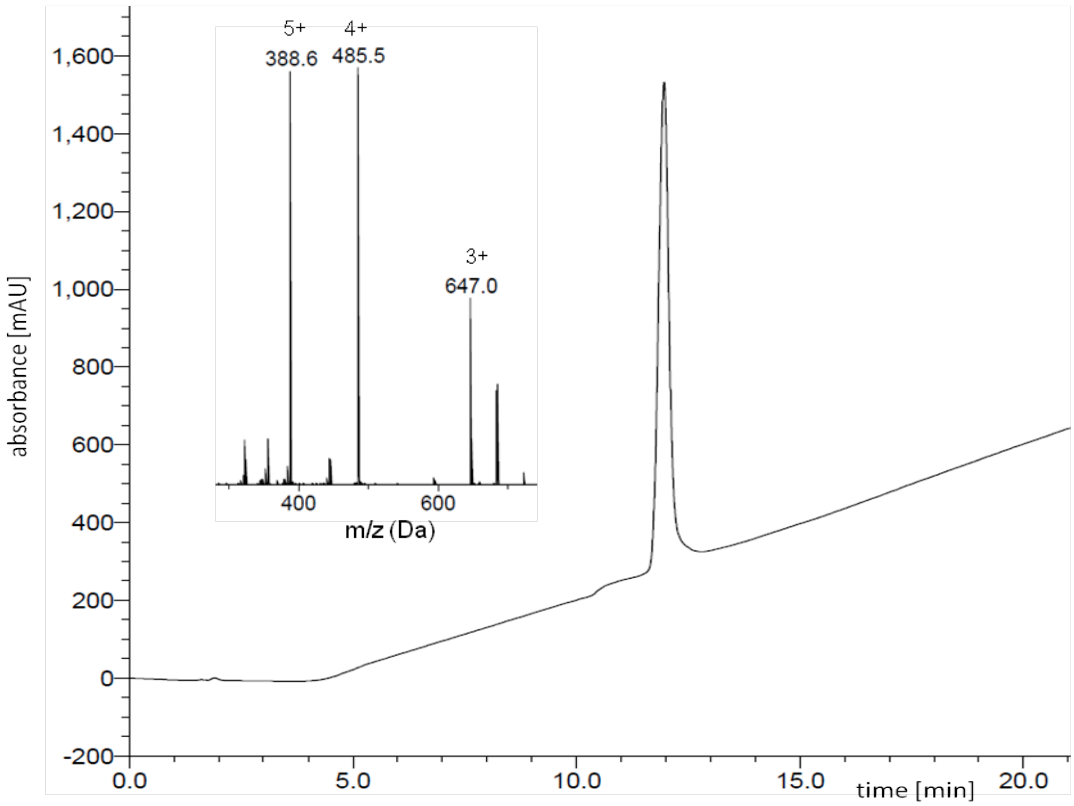
The peptide chain was assembled according to **method 4**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 227 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (3.5 mg, 58%) as a white solid in *ca.* 93% purity according to analytical HPLC (column A). R_t 22.4 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 334.7, observed mass = 334.7; [M+5H$^+$] calculated mass = 401.5, observed mass = 401.4; [M+4H$^+$] calculated mass = 501.6, observed mass = 501.5; [M+3H$^+$] calculated mass = 668.5, observed mass = 668.4.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 3,3-diphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = tetrahydroisoquinoline-3-COOH) (23)

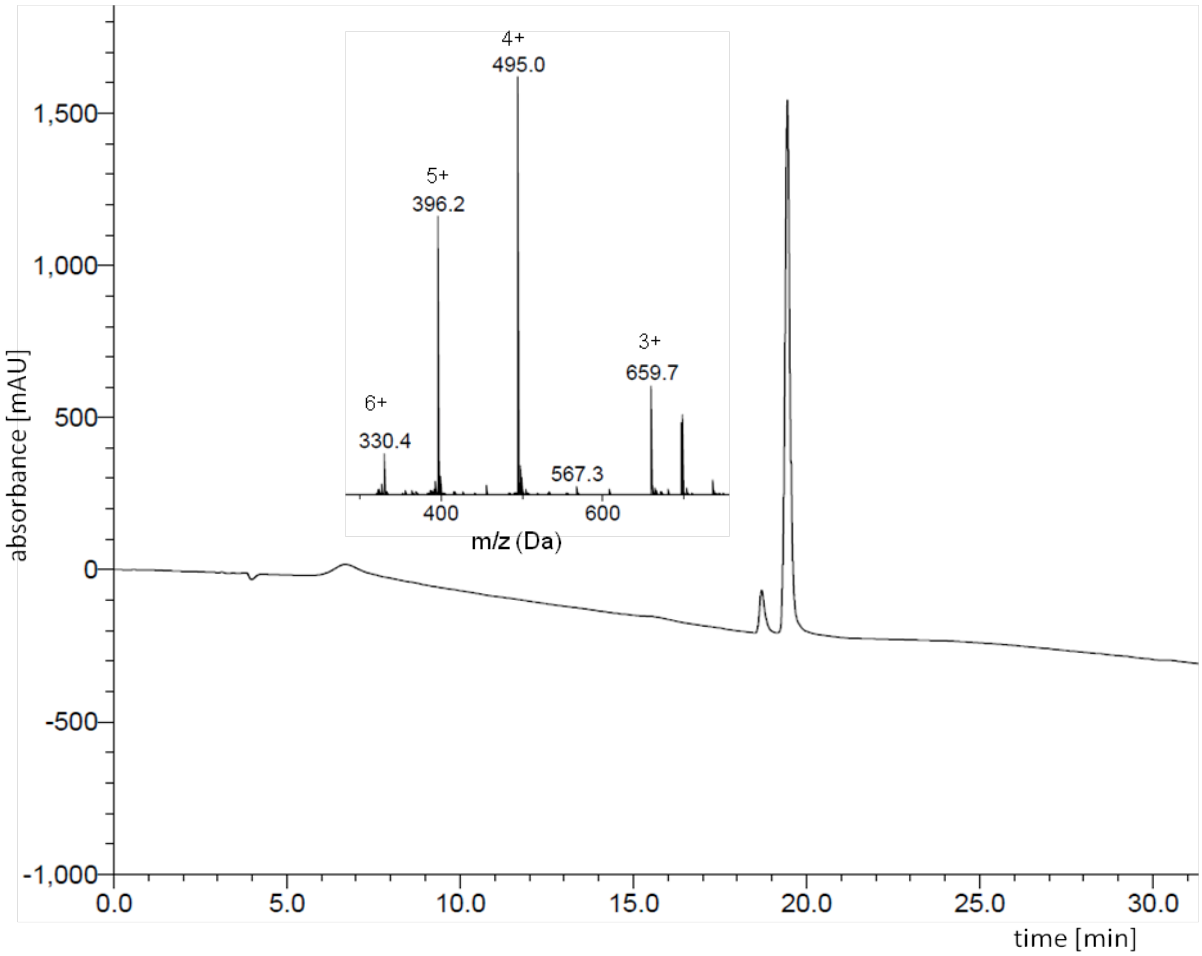
Peptide **24** was synthesized by using a 2-chloro-4'-carboxy-triphenylmethanol[7] linker attached to aminomethyl resin. After chlorination of the trityl alcohol using $SOCl_2$/DCM (1:1, 4 mL, 3h) the C terminal amino acid was attached to the 2-chlorotrityl chloride type resin by esterification using standard procedures.[3] The second amino acid was attached manually to the secondary amine by HATU activation (2 eq AA, 2 eq HATU, 4 eq DIPEA) in 1.5 mL of DMF for 1h followed by HBTU activated coupling of the third residue (4 eq AA, 3.9 eq HBTU, 8 eq NMM) in 1.5 mL of DMF for 1.5 h. The remaining peptide chain was assembled using **method 5**. Capping with biotin according to **method 7** was followed by cleavage according to **method 8** yielding 83 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (5 mg, 23%) as a white solid in *ca.* 98% purity according to analytical HPLC (column A). R_t 12.0 min (Phenomenex Gemini C_{18}, 1-60% B over 20 min, 1 mL/min); *m/z* (ESI-MS): [M+5H$^+$] calculated mass = 388.7, observed mass = 388.6; [M+4H$^+$] calculated mass = 485.6, observed mass = 485.5; [M+3H$^+$] calculated mass = 647.1, observed mass = 647.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = tetrahydroisoquinoline-3-COOH) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 1-naphthylalanine) (24)

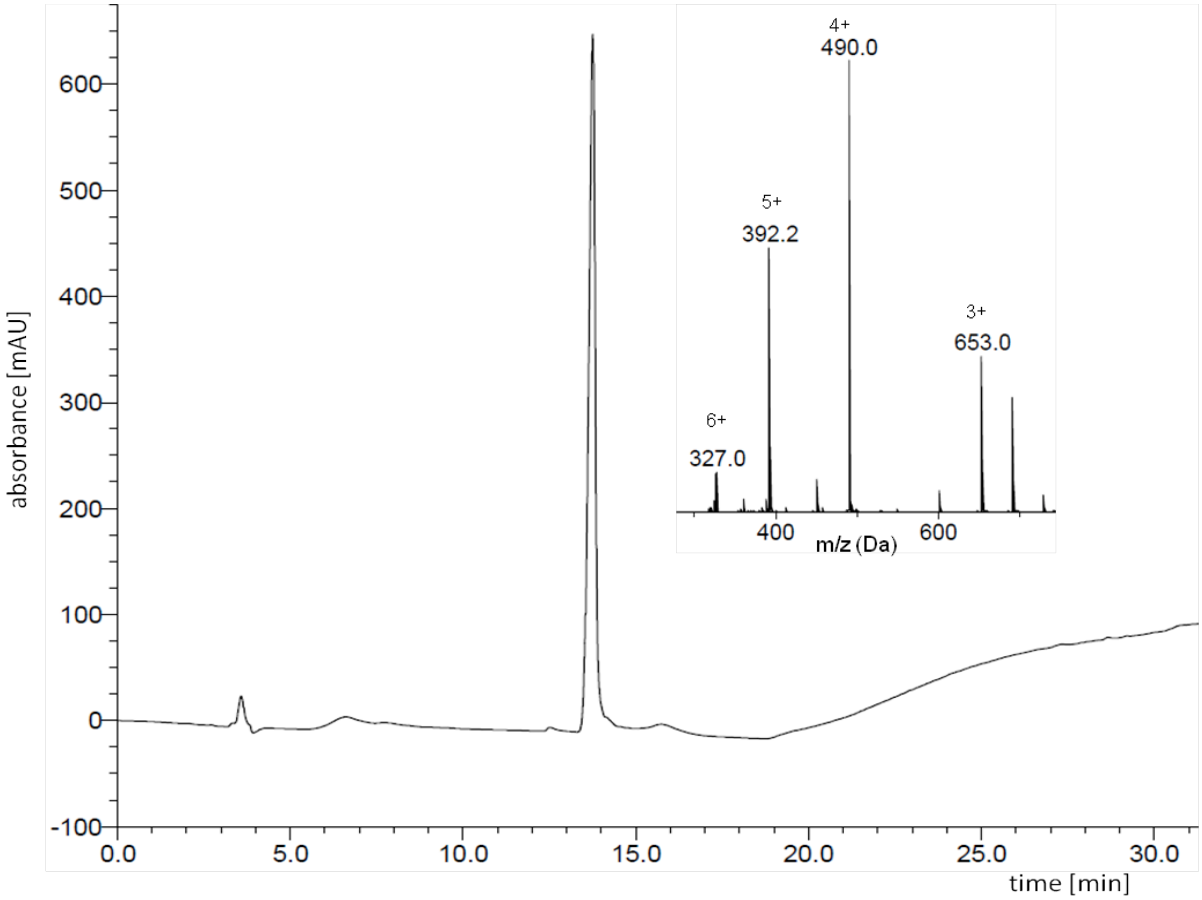
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 123 mg of crude product. 10 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 57%) as a white solid in *ca.* 93% purity according to analytical HPLC (column A). R_t 19.5 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 330.4, observed mass = 330.4; [M+5H$^+$] calculated mass = 396.3, observed mass = 396.2; [M+4H$^+$] calculated mass = 495.1, observed mass = 495.0; [M+3H$^+$] calculated mass = 659.8, observed mass = 659.7.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 1-naphthylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 4-methoxyphenylalanine) (25)

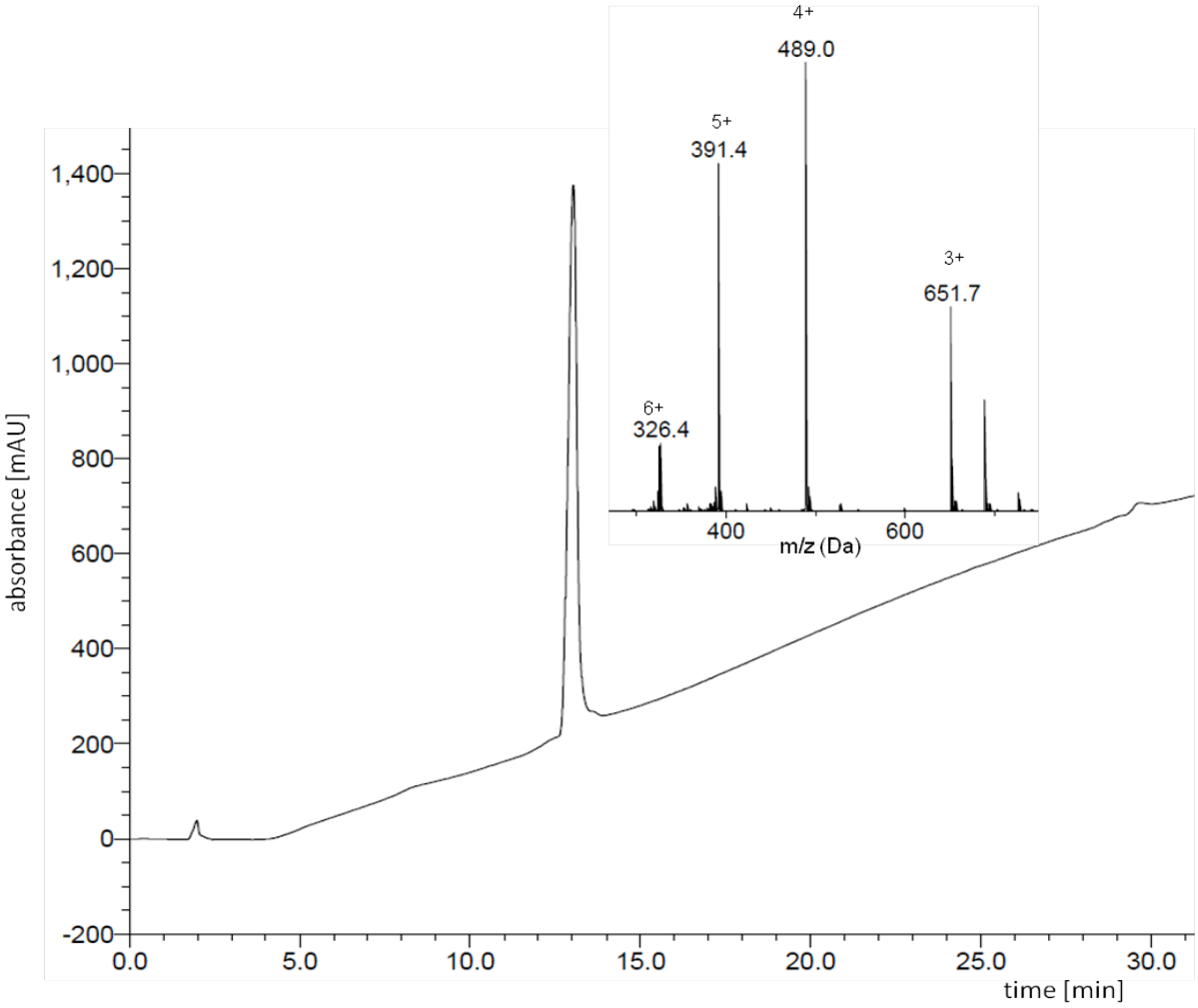
The peptide chain was assembled according to **method 5**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 218 mg of crude product. 14.8 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (2.5 mg, 40%) as a white solid in *ca.* 98% purity according to analytical HPLC (column A). R_t 13.8 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 327.1, observed mass = 327.0; [M+5H$^+$] calculated mass = 392.3, observed mass = 392.2; [M+4H$^+$] calculated mass = 490.1, observed mass = 490.0; [M+3H$^+$] calculated mass = 653.1, observed mass = 653.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 4-methoxyphenylalanine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 2-indanylglycine) (26)

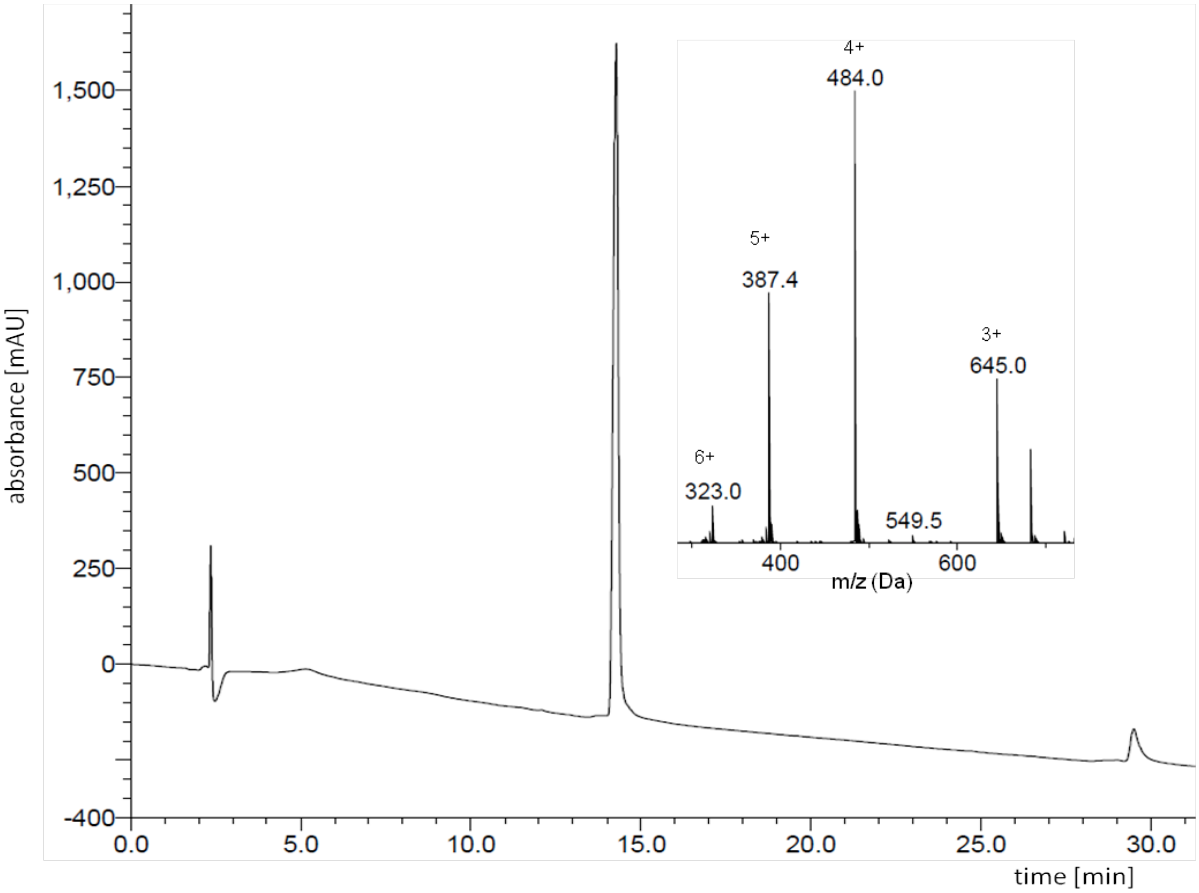
The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 147 mg of crude product. 15.5 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (4 mg, 21%) as a white solid in *ca.* 99% purity according to analytical HPLC (column A). R_t 13.0 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 326.4, observed mass = 326.4; [M+5H$^+$] calculated mass = 391.5, observed mass = 391.4; [M+4H$^+$] calculated mass = 489.1, observed mass = 489.0; [M+3H$^+$] calculated mass = 651.8, observed mass = 651.7.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 2-indanylglycine) including ESI-MS spectrum

biotin-R_8ERY* (Y* = 2-thienylalanine) (27)

The peptide chain was assembled according to **method 6**, capped with biotin according to **method 7** and then cleaved according to **method 8** yielding 127 mg of crude product. 16 mg of crude peptide were purified by semi-preparative HPLC at a flow rate of 5 mL/min using a linear gradient of 1% B to 80 % B over 60 min. Lyophilisation yielded the purified peptide (3.5 mg, 20%) as a white solid in *ca.* 93% purity according to analytical HPLC (column A). R_t 14.3 min (Phenomenex Gemini C_{18}, 1-80% B over 30 min, 1 mL/min); m/z (ESI-MS): [M+6H$^+$] calculated mass = 323.1, observed mass = 323.0; [M+5H$^+$] calculated mass = 387.5, observed mass = 387.4; [M+4H$^+$] calculated mass = 484.1, observed mass = 484.0; [M+3H$^+$] calculated mass = 645.1, observed mass = 645.0.



Analytical HPLC profile (λ = 210 nm) of compound biotin-R_8ERY* (Y* = 2-thienylalanine) including ESI-MS spectrum

P-values of compounds 3-27

	P values for inhibition by biotin-R8ERY* peptides with respect to ERY		
	Peptide	**P value**	
		50 µM	100 µM
27	ERY*2-thienylalanine	0.12	0.04
26	ERY*2-indanylglycine	0.19	0.66
9	ERY* homophenylalanine	0.13	0.51
25	ERY*4-methoxyphenylalanine	0.26	0.09
11	ERF	0.17	0.13
23	ERY* tetrahydroisochinoline-3-COOH	0.23	0.1
15	ERY* 4-cyanophenylalanine	0.25	0.09
3	ERY*4-methyphenylalanine	0.22	0.09
14	ERY* 3-methylphenylalanine	0.88	0.03
15	DRY	0.04	0.16
13	ERY* 4-fluorophenylalanine	0.45	0.15
12	ERY* 4-sulfotyrosine	0.37	0.03
21	ERY* 3-iodophenylalanine	0.16	0.03
20	RY* 3,4-dihydroxyphenylalanine	0.32	0.01
19	EKY* 4,4'-biphenylalanine	0.38	0.01
16	ERY* 3-nitrophenylalanine	0.27	0.02
6	ERY*4-trifluoromethylphenylalanine	0.03	0.02
7	ERY* 2-naphthylalanine	0.15	0.02
5	ERY*4-iodophenylalanine	0.01	0.04
17	ERY* 4-bromophenylalanine	0.61	0.02
18	ERY* 3,4-dichlorophenylalanine	0.14	0.03
24	ERY*1-naphthylalanine	0.01	0.01
22	ERY*3,3-diphenylalanine	0.01	0.002
8	ERY*4,4'-biphenylalamine	0.02	0.01
10	ERY* 4-nitrophenylalanine	0.15	0.004
4	ERY*4-chlorophenylalanine	0.03	0.002

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