Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

A set of rhamnosylation-specific antibodies enables detection of novel protein glycosylations in bacteria

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Material and Methods

Methods and reagents:

If not otherwise noted, all reactions were magnetically stirred and conducted in oven-dried glassware. Reactions were performed in an argon atmosphere using standard Schlenk techniques. Solvents for moisture sensitive reactions (Diethyl ether, Dichloromethane, Tetrahydrofuran) were dried according to standard procedures and distilled prior to use or purchased from Acros Organics as “extra dry” reagents stored over molecular sieve and under inert gas atmosphere (*N,N*-Dimethylformamide (DMF)). Commercially available reagents were purchased from Sigma-Aldrich or TCI-Chemicals and were used without further purification. Analytical thin-layer chromatography (TLC) was used for monitoring of reactions. TLC was performed on pre-coated silica gel 60 F254 aluminium plates (Merck KGaA, Darmstadt) and visualized by exposure to ultraviolet light (UV, 254 nm) and/or staining with either a 1:1 mixture of 1 M H_2SO_4 in EtOH and 3% 4-methoxyphenol solution in EtOH (Carbohydrates) or Seebach reagent (Cerium phosphomolybdic acid (5.0 g) concentrated sulfuric acid (16 ml), water (200 ml) and Cerium(IV) sulphate (2.0 g)). If not otherwise stated, purification of substances was achieved by standard flash column chromatography on silica (35-70 µm particle size) from Acros organics.

NMR spectroscopy:

Proton nuclear magnetic resonance (^{1}H-NMR) were recorded in deuterated solvents ($CDCl_3$, DMSO-d_6) on a Bruker Avance III HD 400 MHz spectrometer equipped with a CyroProbe™, a Varian VXR 400 S spectrometer, a Bruker AMX600 spectrometer or a Bruker Avance III HD 800 MHz spectrometer equipped with a Cyro Probe™. Chemical Shifts (δ scale) were reported in parts per million (ppm) and calibration was carried out with residual protic solvents as internal reference. For indication of multiplicities the following abbreviations were used: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet) as well as combinations thereof. Carbon nuclear magnetic resonance spectra (^{13}C-NMR) were recorded on the aforementioned spectrometers with 100 MHz, 150 MHz and 200 MHz and chemical shifts (δ) were also given in ppm referenced to the central carbon signal of the solvents. For further assignment of the ^{1}H and ^{13}C-NMR signals, 2D-NMR experiments (COSY, HSQC, HMBC) were used. If necessary, anomeric configuration of rhamnose derivatives were proved by using proton coupled HSQC experiments. Numbering of proton and carbon atoms does not necessarily correspond to the IUPAC and are further described in the respective section.

High-resolution mass spectrometry (HRMS):

High resolution (HR-ESI) mass spectra were recorded on a Thermo Finnigan LTQ FT spectrometer either in positive or negative ionization mode.

High performance liquid chromatography (HPLC):

Analytical RP-HPLC was conducted at a JASCO system (PU-2080 Plus, LG-2080-02-S, DG-2080-53 and MD-2010 Plus) on Phenomenex Aeris Peptide column (C18, 5 µm, 250 mm × 4.6 mm; later referred to as "*Aeris*") or Phenomenex Luna column (C18, 5 µm, 250 mm × 4.6 mm; later referred to as "*Luna*"). A gradient of water (A)/acetonitrile (B) containing 0.1% TFA with a flow-rate of 1 ml/min was used as eluent. Semi-preparative RP-HPLC was performed at a JASCO system (PU-2087 Plus, LG-2080-02-S and UV-2075 Plus) on a Phenomenex Aeris Peptide column (C18, 250 × 21.2 mm), with a flow of 16 ml/min. Exact composition of gradients are presented in Table 1 and Table 2 below.

Table 1: Description of gradient A for HPLC-purification

Time [min]	**0**	**40**	**60**
Water [%]	95	20	0
MeCN [%]	5	80	100
TFA [%]	0.1	0.1	0.1
Flow [ml/min]	15	15	15

Table 2: Description of gradient B for HPLC-purification

Time [min]	0	40	60
Water [%]	95	40	0
MeCN [%]	5	60	100
TFA [%]	0.1	0.1	0.1
Flow [ml/min]	15	15	15

Optical rotation:

Optical rotations were measured on a Perkin-Elmer polarimeter 241 at the Sodium-D-line (589 nm) at the given temperature in °C. Concentration c is given in g/100 ml in the solvent stated in brackets ($CHCl_3$).

Microwave assisted peptide synthesis (for unglycosylated peptides (naked peptides NP); 0.1 mmol scale)

A pre-loaded Gly-Wang resin (Rapp Polymere) with a loading of 0.66 mmol/g was swelled 0.5 h in 5 ml DMF prior to use. DIC/OXYMA (0.5 M in DMF) and DIPEA (0.1 M in DMF) were used as coupling reagents. Fmoc-protected amino acids (Fmoc-Asn(Trt)-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Gly-OH and Fmoc-Ile-OH from Orpegen Pharma or Sigma Aldrich) as well as the Fmoc-TEG-CO_2H-Spacer were coupled with a double coupling protocol (2 × coupling protocol: 75 °C, 170 W, 15 sec → 90 °C, 30 W, 120 sec, 5.0 eq of Fmoc-AA-OH per coupling). Deprotection of the Fmoc-protecting group was achieved by treatment with 20 % piperidine in DMF (2 × deprotection protocol: 75 °C, 155 W, 15 sec → 90 °C, 30 W, 50 sec). Upon completion of the solid-phase peptide synthesis, the resin-bound peptide was cleaved from the resin by treatment with TFA/H_2O/Tri-*iso*-propylsilane (98:1:1) for 1 h. After filtration, the crude product was precipitated from 40 ml of cold diethyl ether. The crude peptide was collected by centrifugation and the supernatant was discarded. The peptide was washed with cold diethyl ether and dried under a flow of nitrogen. The crude residue was dissolved in water and lyophilized prior to purification by RP-HPLC.

Conventional peptide synthesis (rhamnosylated glycopeptides)

a) Resin swelling:

H-Gly-2-ClTrt resin (Loading: 1.1 mmol/g) in a 2 ml SPPS vessel was added 2 ml DMF and was shaken for 30 min at room temperature.

b) Coupling of non-glycosylated amino acid building blocks:

Synthesis on a 0.1 mmol scale:
To a solution of Fmoc aa-OH (4.0 eq.) in 455 µl DMF was added a 1 M PyBOP solution in DMF (400 µl, 4.0 eq.), a 1 M HOBT·H_2O solution in DMF (400 µl, 4.0 eq.) and a 2 M DIPEA solution in DMF (400 µl, 8.0 eq.). The solution was shaken for 1 minute before being added to the resin. The mixture was agitated at room temperature (1 h per coupling for Gly, 1.5 h per coupling for Ile). For Gly a double coupling protocol was applied, Ile was coupled *via* a single coupling protocol. After each coupling step, the resin was filtered and washed with DMF (5 × 2 ml).

Synthesis on a 0.05 mmol scale:
To a solution of Fmoc aa-OH (8.0 eq.) in 455 µl DMF was added a 1 M PyBOP solution in DMF (400 µl, 8.0 eq.), a 1 M HOBT· H_2O solution in DMF (400 µl, 8.0 eq.) and a 2 M DIPEA solution in DMF (400 µl, 16.0 eq.). The solution was shaken for 1 minute before being added to the resin. The mixture was agitated at room temperature (1 h per coupling for Gly, 1.5 h per coupling for Ile). For Gly a double coupling protocol was applied, Ile was coupled *via* a single coupling protocol. Subsequently, the resin was filtered and washed with DMF (5 × 2 ml).

c) Coupling of rhamnosyl amino acid building blocks (1, 2, 4, 5, 7 and 9) and Fmoc-TEG-CO_2H Spacer:

For the incorporation of the rhamnosylated amino acid blocks, as well as the Fmoc-TEG-CO_2H Spacer a double coupling protocol was used. Conditions for both coupling steps can be found in the corresponding section below.

d) Deprotection of rhamnosylated glycopeptides[1] and cleavage from resin

Removal of the acetyl protecting groups was accomplished by addition of 2 ml of a 5% N_2H_2 solution in DMF to the resin-bound peptide. The mixture was agitated for 17 h at room temperature. Subsequently, the resin was filtered and washed with DMF (5 × 2 ml), MeOH (5 × 2 ml) and CH_2Cl_2 (5 × 2 ml). Cleavage from resin was conducted by treatment with 1 ml of a mixture of TFA/H_2O/Tri-*iso*-propylsilane (98:1:1). The solution was concentrated under a flow of Nitrogen before being added dropwise to chilled Et_2O for precipitation of the crude peptide. The mixture was centrifuged and the supernatant was discarded. The crude peptide was dissolved in water and lyophilized prior to purification by RP-HPLC.

Assignment of peptide NMR signals:

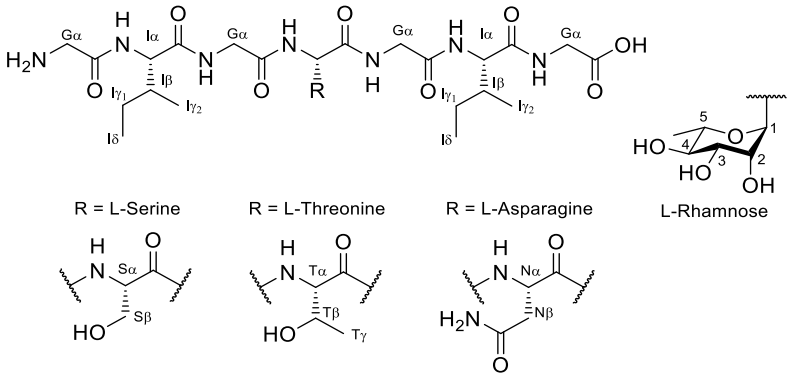


Figure 1: Assignment of Peptide NMR-Signals.

Structure and abbreviation of TEG-Spacer for BSA conjugation:

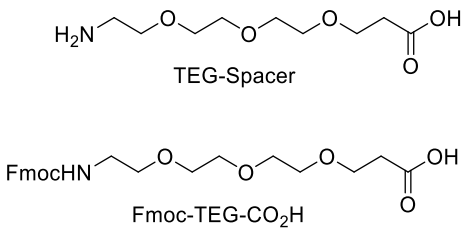


Figure 2: Chemical structures of TEG-spacer derivatives and abbreviations used in the following.

Antibody generation and purification

The polyclonal antibodies (*anti*-Asn^{Rha}, *anti*-Ser^{Rha}, *anti*-Thr^{Rha}) were commercially produced by Eurogentec using the Rabbit Speedy 28-day (AS superantigen) program. Two rabbits were immunized per antibody production by multi-site (front right shoulder, front left shoulder, right thigh, left thigh) subcutaneous injections (4 injections: day 0, 7, 10, 18) with mono-rhamnosylated peptides coupled to Keyhole limpet hemocyanin (KLH).

Per injection 500 µl Freund´s adjuvant (incomplete) were administered together with 500 µl antigen with a concentration of 400 µg/ml (equimolar mixtures of the corresponding α- and β-rhamnosylated glycopeptide, 200 µg in total per rabbit). The final bleed was taken after 28 days and provided 70 ml serum. The polyclonal antibodies were extracted from the serum by affinity chromatography (AS-PURI-MED). Therefore, the corresponding glycopeptide was coupled to a matrix (AF-Amino TOYO). The serum was loaded, and unrelated antibodies were washed off. Antibodies recognizing the rhamnosylated peptides were eluted and the affinity of

antibodies was analysed by indirect Enzyme-linked Immunosorbent Assay (ELISA). The plate was coated with the corresponding glycopeptide (100 ng/well) and carrier protein KLH as control. Different dilutions of the serum, flow through, purified antibody and pre-immune sera were tested. The optical density of the chromogenic substrate (secondary HRP-conjugated antibody, chromogenic substrate *o*-phenylenediamine) was measured at 492 nm (OD492). For all purified antibodies, sigmoidal ELISA curves were observed for the glycopeptide. Final analysis with BIOANALYSER (Agilent) revealed 89.4 %, 68.3 % and 84.6 % antibody purity for *anti*-Asn^{Rha}, *anti*-Ser^{Rha} and *anti*-Thr^{Rha}, respectively.

SDS-PAGE and Western Blotting

Electrophoretic separation of proteins was carried out using SDS-PAGE (**s**odium **d**odecyl **s**ulphate **p**oly**a**crylamide **g**el **e**lectrophoresis) as described by Lämmli[2]. Specifically, the stacking gel contained 4.8 v% acrylamide (Roth) and the separating gel contained 12.5 v% acrylamide. Here, 0.5 v% 2-2-2-trichloroethanol was added for protein visualization using the Gel Doc EZ Gel Documentation System (Bio-Rad). Polymerization of the gels was induced using APS and TEMED. By default, samples were mixed with 5X SDS loading dye (250 mM Tris, 0.1 g/mL SDS, 5 mg/mL BPP, 50 v% glycerol, 500 mM DTT, pH 6.8), heated to 95 °C for 10 min and subsequently, 15 to 20 µL were loaded per lane. ROTI®Mark 10-150 (Roth) was used as size standard. Proteins were separated using PerfectBlue SDS-PAGE chambers (Peqlab) filled with Lämmli buffer (25 mM Tris, 200 mM glycine, 1 g/L SDS, pH 8.2 - 8.3) at 200 V.

Separated proteins were visualized in gel according to Ladner *et al.*[3] or using InstantBlue™ (Expedeon Ltd.). For Western Blot analyses SDS-PAGE separated proteins were transferred to a nitrocellulose membrane by vertical Western Blotting. Antigens were detected using 0.2 µg/ml *anti*-Arg^{Rha}, 0.2 µg/ml *anti*-Asn^{Rha}, 0.2 µg/ml *anti*-Ser^{Rha} and 0.2 µg/ml *anti*-Thr^{Rha}. Primary antibodies (rabbit) were targeted with 0.1 µg/ml anti-rabbit IgG (IRDye® 680RD) (donkey) antibodies (abcam). Target proteins were visualized *via* the Odyssey® CLx Imaging System (LI-COR, Inc).

For the calculation of the molecular weight (MW) of BSA, BSA coupled to naked peptide (BSA-aa^{NP}) and BSA conjugates with the corresponding rhamnosylated glycopeptides (BSA-aa$^{\alpha\text{-}/\beta\text{-}Rha}$) from SDS-PAGE gels a graph of log MW vs. relative migration distance (R_f) was plotted, based on the values obtained for the bands in the MW standard (ROTI®Mark 10-150 (Roth)). The MW band was then calculated by interpolation using this graph.

Construction of plasmids for sensitivity and specificity analysis

In order to obtain EF-P variants comprising the peptide sequence of the naked (unmodified) peptides (NP) (Gly-Ile-Gly-Asn-Gly-Ile-Gly (Asn^{NP}), Gly-Ile-Gly-Ser-Gly-Ile-Gly (Ser^{NP}) and Gly-Ile-Gly-Thr-Gly-Ile-Gly (Thr^{NP})) at its loop region (EF-P^{Asn-NP}, EF-P^{Ser-NP}, EF-P^{Thr-NP}) the corresponding peptide sequence were introduced into His_6-tagged $efp_{P.pu}$ (pBAD24-$efp_{P.pu}$)[4] by overlap extension PCR.[5] Oligonucleotides used in this study are listed in table 3. Kits were used according to the manufacturers' directions. Plasmid DNA was isolated using a Hi Yield plasmid minikit (Süd-Laborbedarf GmbH), DNA fragments were purified by employing a Hi Yield PCR clean-up (Süd-Laborbedarf). Sequence amplifications by PCR were performed utilizing the Q5 high-fidelity DNA polymerase (NEB). All constructs were analysed by Sanger sequencing (LMU Sequencing Service). Standard methods were performed according to the instructions of in the literature.[6]

Table 3: Oligonucleotides for plasmid construction

Oligonucleotide	Sequence (5'-3')
Seq33_fw	GGC GTC ACA CTT TGC TAT GC
pBAD-HisA_rev	CAG TTC CCT ACT CTC GCA TG
	GGC ATT GGC AAC GGC ATT GGC ATC ATG
EF-P(Ppu)synL_Asn_OL_Fw	AAG ACC AAG CTG AAG AAC C
	GCC AAT GCC GTT GCC AAT GCC GGT GAA
EF-P(Ppu)synL_Asn_OL_Rev	CTC AGC TTT TTG AAC CAG
EF-P(Ppu)synL_Thr_OL_Fw	GGC ATT GGC ACC GGC ATT GGC ATC ATG
	AAG ACC AAG CTG AAG AAC C
	GCC AAT GCC GGT GCC AAT GCC GGT GAA
EF-P(Ppu)synL_Thr_OL_Rev	CTC AGC TTT TTG AAC CAG
	GGC ATT GGC AGC GGC ATT GGC ATC ATG
EF-P(Ppu)synL_Ser_OL_Fw	AAG ACC AAG CTG AAG AAC C
	GCC AAT GCC GCT GCC AAT GCC GGT GAA
EF-P(Ppu)synL_Ser_OL_Rev	CTC AGC TTT TTG AAC CAG

Protein production and purification

The His_6-tagged EF-$P_{P.pu}$ containing the peptide sequence of the naked peptides was transformed in chemically competent *E. coli* LMG194 as strain for protein production. After promotor induction with arabinose (0.2 % (wt/vol)), cells were incubated at 37 °C for 3 h and lysed afterwards by sonication. The His_6-tagged protein was purified using Ni-nitrilotriacetic acid (Ni-NTA; Qiagen) according to the manufacturer's instructions.

Synthesis and characterization of rhamnosylated Amino acid building blocks:

Synthesis of *O*-rhamnosyl serine and threonine SPPS building blocks:

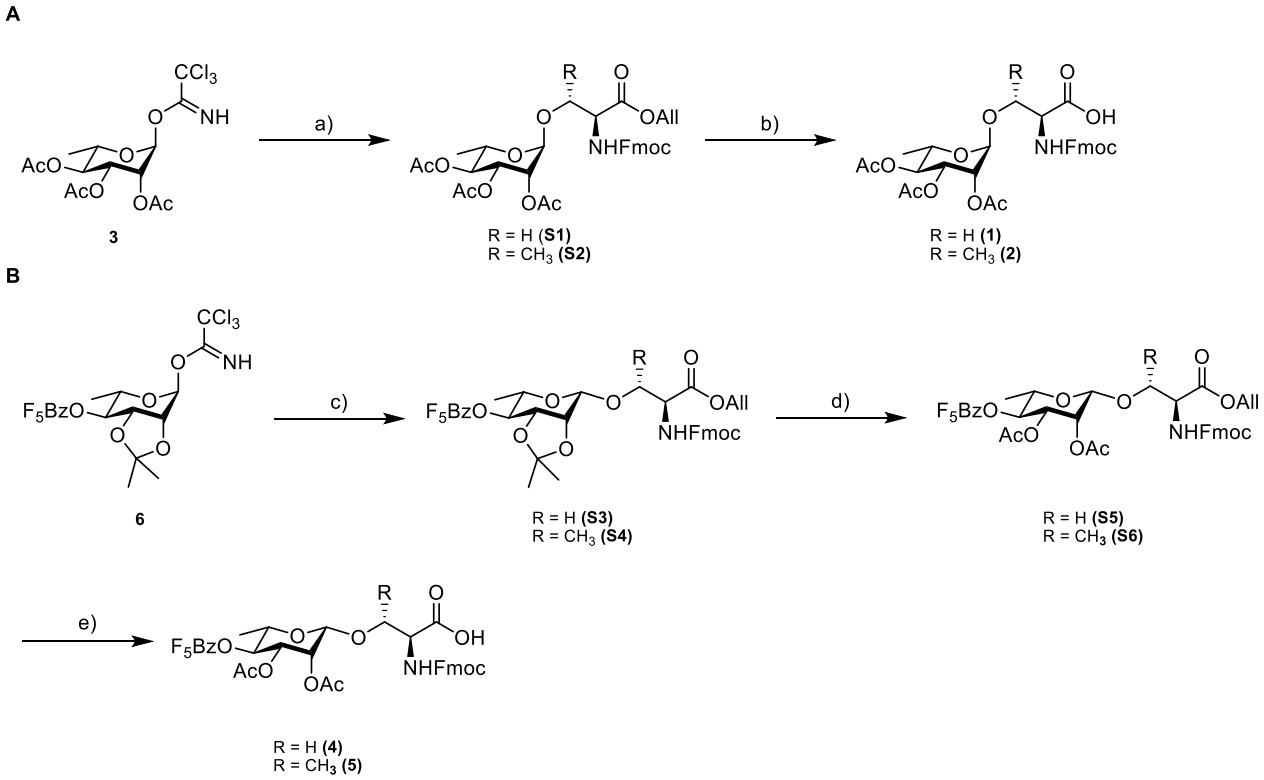
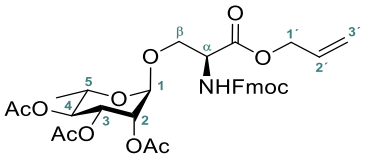


Figure 3: General overview of the chemical synthesis of *O*- rhamnosylated serine and threonine SPPS building blocks. **A**) Synthesis of α-*O*-rhamnosyl serine and threonine building blocks: Reagents and conditions: a) Serine: Fmoc-Ser-OAll, TMSOTf, CH_2Cl_2, 74%; Threonine: Fmoc-Thr-OAll, TMSOTf, CH_2Cl_2, 75%; b) $PhSiH_3$, $[Pd(PPh_3)_4]$, CH_2Cl_2, 97% (**1**), 85% (**2**). **B**) Synthesis of β-*O*-rhamnosyl serine and threonine building blocks: Reagents and Conditions: Serine: Fmoc-Ser-OAll, $HNTf_2$, Et_2O/CH_2Cl_2 76%; threonine: Fmoc-Thr-OAll, $HNTf_2$, Et_2O/CH_2Cl_2 85%; d) 1) TFA, H_2O, Ac_2O, pyridine 87% (**S5**), 74% (**S6**) over 2 steps; e) $[Pd(PPh_3)_4]$, *N*-methylaniline, THF, 98% (**4**), 94% (**5**).

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-serine allyl ester (**S1**)



2,3,4-Tri-*O*-acetyl-L-rhamnopyranosyl trichloroacetimidate **3**[7] (2.01 g, 4.63 mmol, 1.7 eq.) and Fmoc-Ser-OAll[8] (1.00 g, 2.72 mmol, 1.0 eq.) were combined and co-evaporated with toluene (2 × 10 ml). The mixture was dried 1 h under high vacuum before being dissolved in dry CH_2Cl_2 (35 ml). Freshly activated 4 Å molecular sieve was added and the reaction mixture was stirred for 1 h at room temperature before being chilled to 0 °C. Subsequently, TMSOTf (54.0 µl, 0.30 mmol, 0.1 eq.) was added in one portion and the stirring was continued at 0 °C until complete conversion of the acceptor was detected by TLC monitoring. The reaction was stopped by addition of NEt_3 (200 µl) filtered through a pad of *Hyflo* and concentrated to dryness under reduced pressure. The crude product was directly subjected to flash chromatography (^{c}Hex/EtOAc v/v = 3:1) affording **S1** (1.29 g, 2.02 mmol, 74%) as a colourless foam.

R$_f$ = 0.52 (^{c}Hex/EtOAc v/v = 1:1).

Optical rotation $[\alpha]_D^{22}$ = − 18.0° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.79 – 7.73 (m, 2H, 2 × CH-Fmoc), 7.65 (d, $J_{CH,CH}$ = 6.4 Hz, 2H, 2 × CH-Fmoc), 7.42 – 7.38 (m, 2H, 2 × CH-Fmoc), 7.37 – 7.30 (m, 2H, 2 × CH-Fmoc), 5.97 (ddt, $J_{H2´,H3trans}$ = 16.6 Hz, $J_{H2´,H3cis}$ = 10.3 Hz, $J_{H2´,H1}$ = 6.0 Hz, 1H, H-2´), 5.71 (d, $J_{NH,Hα}$ = 8.6 Hz, 1H, NH-Fmoc), 5.39 (d, $J_{H3´trans,\ H2´}$ = 17.1 Hz, 1H, H-3´$_{trans}$), 5.32 – 5.27 (m, 2H, H-2, H-3´$_{cis}$), 5.17 (dd, $J_{H3,H4}$ = 10.1 Hz, $J_{H3,H2}$ = 3.5 Hz, 1H, H-3), 5.06 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 10.0 Hz, 1H, H-4), 4.75 (s, 1H, H-1), 4.71 (d, $J_{H1´,H2´}$ = 6.0 Hz, 2H, H-1´), 4.64 (dt, $J_{Hα,NH}$ = 8.8 Hz, $J_{Hα,Hβ}$ = 3.1 Hz, 1H, H-α), 4.45 – 4.37 (m, 2H, CH_2-Fmoc), 4.27 (t, $J_{CH,CH}$ = 7.3 Hz, 1H, CH-Fmoc), 4.19 (dd, $J_{Hβ,Hβ´}$ = 9.9 Hz, $J_{Hβ,Hα}$ = 3.1 Hz, 1H, H-β), 3.76 (m, 1H, H-5), 3.70 (dd, $J_{Hβ´,Hβ}$ = 9.9 Hz, $J_{Hβ´,Hα}$ = 3.1 Hz, 1H, H-β´), 2.15 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.21 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.2 (C=O), 170.0 (2C, 2 × C=O), 169.4 (C=O), 156.1 (Fmoc-C=O), 144.0 (Cq-Fmoc), 143.9 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 131.4 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.4 (2C, 2 × CH-Fmoc), 120.1 (2C,

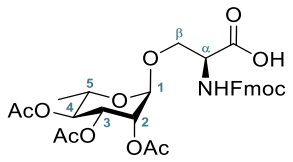
2 × CH-Fmoc), 119.8 (C-3´), 97.7 (C-1), 71.0 (C-4), 69.6 (C-2), 69.0 (C-3), 68.1 (C-β), 67.6 (CH_2-Fmoc), 66.9 (C-5), 66.7 (C-1´), 54.3 (C-α), 47.3 (CH-Fmoc), 21.0 (OAc), 20.9 (OAc), 20.8 (OAc), 17.5 (rha-CH_3). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 175 Hz.

HRMS (ESI+): Calculated for $C_{33}H_{41}O_{12}N_2$ $[M+NH_4]^+$: 657.2654; found: 657.2670.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 18.55 min, λ = 230 nm.

N^{α}-Fluorenylmethoxycarbonyl-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-serine (**1**)



Cleavage of the allylic ester was conducted according to a slightly modified procedure.[9]

To a magnetically stirred solution of rhamnosyl-serine derivative **S1** (1.00 g, 1.56 mmol, 1.0 eq.) in dry CH_2Cl_2 (25 ml), Phenylsilane (427 µl, 3.43 mmol, 2.2 eq.) and $[Pd(PPh_3)_4]$ (36.0 mg, 31.3 µmol, 0.02 eq.) were added. The reaction was stirred at ambient temperature until complete conversion of the starting material was observed by TLC monitoring. The reaction was quenched by addition of water (1.50 ml) and concentrated to dryness under reduced pressure. The crude residue was co-evaporated with toluene (2 × 15 ml) and directly subjected to flash chromatography (^{c}Hex/EtOAc v/v = 2:1 + 1% AcOH → 1:1 + AcOH) affording **1** (911 mg, 1.52 mmol, 97%) as a colourless foam.

R_f = 0.22 (^{c}Hex/EtOAc v/v = 1:1 + 1% AcOH).

^{1}H-NMR: (400 MHz, $CDCl_3$): δ = 7.76 (dd, $J_{CH,CH}$ = 7.6 Hz, $J_{CH,CH}$ = 2.5 Hz, 2H, 2 × CH-Fmoc), 7.66 – 7.60 (m, 2H, 2 × CH-Fmoc), 7.39 (t, $J_{CH,CH}$ = 7.6 Hz, 1H, 2 × CH-Fmoc), 7.32 (td, $J_{CH,CH}$ = 7.6 Hz, $J_{CH,CH}$ = 2.9 Hz, 2H, 2 × CH-Fmoc), 5.97 (d, $J_{NH,Hα}$ = 8.5 Hz, 1H, NH-Fmoc), 5.32 (dd, $J_{H2,H3}$ = 3.5 Hz, $J_{H2,H1}$ = 1.7 Hz, 1H, H-2), 5.21 (dd, $J_{H3,H4}$ = 10.2 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 5.05 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.9 Hz, 1H, H-4), 4.78 (s, 1H, H-1), 4.69 (dt, $J_{Hα,NH}$ = 8.6 Hz,

$J_{H\alpha,H\beta}$ = 3.3 Hz, 1H, H-α), 4.45 – 4.36 (m, 2H, CH_2-Fmoc), 4.28 – 4.17 (m, 2H, CH-Fmoc, H-β), 3.88 – 3.81 (m, 1H, H-5), 3.75 (dd, $J_{H\beta',H\beta}$ = 10.2 Hz, $J_{H\beta',H\alpha}$ = 3.3 Hz, 1H, H-β´), 2.16 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.21 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm]

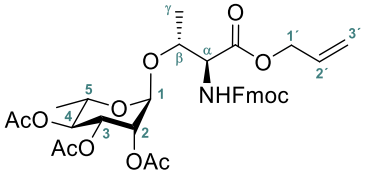
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 172.2 (C=O), 170.5 (C=O), 170.4 (C=O), 170.2 (C=O), 156.3 (Fmoc-C=O), 143.9 (Cq-Fmoc), 143.8 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.4 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 97.8 (C-1), 71.0 (C-4), 69.7 (C-2), 69.3 (C-3), 67.9 (C-β), 67.7 (CH_2-Fmoc), 67.0 (C-5), 54.0 (C-α), 47.2 (CH-Fmoc), 21.1 (OAc), 20.9 (2C, 2 × OAc), 17.4 (rha-CH_3). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 175 Hz.

HRMS (ESI+): Calculated for $C_{30}H_{37}O_{12}N_2$ $[M+NH_4]^+$: 617.2341; found: 617.2347.

For further analytical data see reference.[10, 11]

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-threonine allyl ester (**S2**)



2,3,4-tri-*O*-acetyl-L-rhamnopyranosyl trichloroacetimidate **3**[7] (1.94 g, 4.46 mmol, 1.7 eq) and Fmoc-Thr-OAll[8] (1.00 g, 2.62 mmol, 1.0 eq.) were combined and co-evaporated with toluene (2 × 10 ml). The mixture was dried 1 h under high vacuum before being dissolved in dry CH_2Cl_2 (35 ml). Freshly activated 4 Å molecular sieve was added and the reaction mixture was stirred for 1 h at room temperature, before being chilled to 0 °C. Subsequently, TMSOTf (54.0 µl, 0.30 mmol, 0.1 eq.) was added in one portion and the stirring was continued at 0 °C until complete conversion of the acceptor was detected by TLC monitoring. The reaction was stopped by addition of NEt_3 (200 µl) filtered through a pad of *Hyflo* and concentrated to dryness under reduced pressure. The crude product was directly subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1) affording **S2** (1.29 g, 1.97 mmol, 75%) as a colourless oil.

R_f = 0.17 (^{c}Hex/EtOAc v/v = 3:1).

Optical rotation: $[\alpha]_D^{24}$ = − 47.4° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (400 MHz, $CDCl_3$): δ = 7.77 (d, $J_{CH,CH}$ = 7.8 Hz, 2H, 2 × CH-Fmoc), 7.69 – 7.62 (m, 2H, 2 × CH-Fmoc), 7.41 (td, $J_{CH,CH}$ = 7.2 Hz, $J_{CH,CH}$ = 2.4 Hz, 2H, 2 × CH-Fmoc), 7.39 – 7.29 (m, 2H, 2 × CH-Fmoc), 5.96 (ddt, $J_{H2´,H3´trans}$ = 16.5 Hz, $J_{H2´,H3´cis}$ = 10.4 Hz, $J_{H2´,H1´}$ = 6.0 Hz, 1H, H-2´), 5.56 (d, $J_{NH,Hα}$ = 9.8 Hz, 1H, NH-Fmoc), 5.37 (dt, $J_{H3´trans,\ H2´}$ = 17.2 Hz, $J_{H3´trans,\ H1´}$ = 1.5 Hz, 1H, H-3´$_{trans}$), 5.31 – 5.26 (m, 1H, H-3´$_{cis}$), 5.22 (dd, $J_{H2,H3}$ = 3.5 Hz, $J_{H2,H1}$ = 1.7 Hz, 1H, H-2), 5.16 (dd, $J_{H3,H4}$ = 10.1 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 5.05 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.8 Hz, 1H, H-4), 4.84 (d, $J_{H1,H2}$ = 1.8 Hz, 1H, H-1), 4.74 (ddt, $J_{CH,CH}$ = 13.0 Hz, $J_{H1´,H2´}$ = 5.9 Hz, $J_{H1´,H3cis´}$ = $J_{H1´,H3´trans}$ = 1.3 Hz, 1H, H-1´), 4.63 (ddt, $J_{CH,CH}$ = 12.9 Hz, $J_{H1´,H2´}$ = 6.0 Hz, $J_{H1´,H3cis´}$ = $J_{H1´,H3´trans}$ = 1.3 Hz, 1H, H-1´), 4.53 (dd, $J_{Hα,NH}$ = 9.8 Hz, $J_{Hα,Hβ}$ = 2.4 Hz, 1H, H-α), 4.49 – 4.35 (m, 3H, H-β, CH_2-Fmoc), 4.28 (t, $J_{CH,CH}$ = 7.2 Hz, 1H, CH-Fmoc), 3.73 (dq, $J_{H5,H4}$ = 10.0 Hz, $J_{H5,CH3}$ = 6.3 Hz, 1H, H-5), 2.16 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.26 (d, $J_{Hγ,Hβ}$ = 6.1 Hz, 3H, H-γ), 1.20 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm]

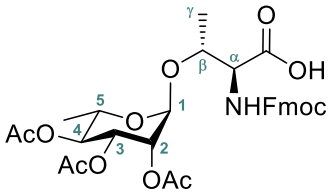
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.3 (C=O), 170.1 (C=O), 170.0 (2C, 2 × C=O), 156.8 (Fmoc-C=O), 144.0 (Cq-Fmoc), 143.8 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 131.4 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.4 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 119.6 (C-3´), 94.2 (C-1), 71.9 (C-β), 70.9 (C-4), 70.1 (C-2), 69.1 (C-3), 67.7 (CH_2-Fmoc), 67.1 (C-5), 66.6 (C-1´), 58.7 (C-α), 47.3 (CH-Fmoc), 21.0 (OAc), 20.9 (2C, 2 × OAc), 17.5 (rha-CH_3), 15.1 (Cγ). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 172 Hz.

HRMS (ESI+): Calculated for $C_{34}H_{39}O_{12}NK$ $[M+K]^+$: 692.2104; found: 692.2103.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 16.55 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-threonine (**2**)



Cleavage of the allylic ester was conducted according to a slightly modified procedure.[9]

Rhamnosyl amino acid **S2** (1.15 g, 1.76 mmol, 1.0 eq) was dissolved in dry CH_2Cl_2 (30 ml), before Phenylsilane (482 µl, 3.87 mmol, 2.2 eq) and [$Pd(PPh_3)_4$] (41.0 mg, 35.2 µmol, 0.02 eq) were added. The reaction was stirred at ambient temperature until complete conversion of the starting material was observed by TLC monitoring. The reaction was stopped by addition of water (0.5 ml) and concentrated to dryness. The crude residue was co-evaporated with toluene (2 × 25 ml) and directly subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 2:1 + 1% AcOH → 1:1 + 1% AcOH) affording **2** (920 mg, 1.50 mmol, 85%) as a colourless foam.

R$_f$ = 0.29 (^{c}Hex/EtOAc v/v = 1:1 + 1% AcOH).

^{1}H-NMR: (400 MHz, $CDCl_3$): δ = 7.77 (d, $J_{CH,CH}$ = 7.9 Hz, 2H, 2 × CH-Fmoc), 7.64 (t, $J_{CH,CH}$ = 6.5 Hz 2H, 2 × CH-Fmoc), 7.40 (td, $J_{CH,CH}$ = 7.2 Hz, $J_{CH,CH}$ = 2.7 Hz, 2H, 2 × CH-Fmoc), 7.37 – 7.30 (m, 2H, 2 × CH-Fmoc), 5.67 (d, $J_{NH,H\alpha}$ = 9.7 Hz, 1H, NH-Fmoc), 5.23 (dd, $J_{H2,H3}$ = 3.5 Hz, $J_{H2,H1}$ =1.7 Hz, 1H, H-2), 5.18 (dd, $J_{H3,H4}$ = 10.0 Hz, $J_{H3,H2}$ = 3.5 Hz, 1H, H-3), 5.04 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.9 Hz, 1H, H-4), 4.85 (d, $J_{H1,H2}$ = 1.8 Hz, 1H, H-1), 4.55 (dd, $J_{H\alpha,NH}$ = 9.7 Hz, $J_{H\alpha,H\beta}$ = 2.4 Hz, 1H, H-α), 4.53 – 4.36 (m, 3H, CH_2-Fmoc, H-β), 4.26 (t, $J_{CH,CH}$ = 7.2 Hz, 1H, CH-Fmoc), 3.89 – 3.76 (m, 1H, H-5), 2.16 (s, 3H, OAc), 1.99 (s, 6H, 2 × OAc), 1.26 (d, $J_{H\gamma,H\beta}$ = 6.2 Hz, 3H, H-γ), 1.18 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm]

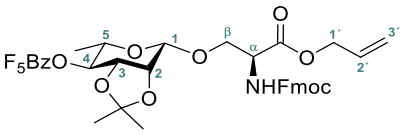
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 173.5 (C=O), 170.4 (2C, C=O), 170.2 (C=O), 156.9 (Fmoc-C=O), 143.9 (Cq-Fmoc), 143.8 (Cq-Fmoc), 141.4 (2C, 2 ×Cq-Fmoc), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.4 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 94.4 (C-1), 71.9 (C-β), 70.8 (C-4), 70.2 (C-2), 69.3 (C-3), 67.8 (CH_2-Fmoc), 67.3 (C-5), 58.3 (C-α), 47.2 (CH-Fmoc), 21.1 (OAc), 20.9 (2C, 2 × OAc), 17.3 (rha-CH_3), 15.2 (C-γ). [ppm].

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 172 Hz.

HRMS (ESI+): Calculated for $C_{31}H_{35}O_{12}NK$ $[M+K]^+$: 652.1791; found: 652.1794.

Further analytical data see reference.[11]

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-Isopropylidene-4-*O*-pentafluorobenzoyl-β-L-rhamnopyranosyl)-L-serine allyl ester (**S3**)



2,3-*O*-Isopropylidene-4-*O*-pentafluorobenzoyl-α-L-rhamnosyl trichloroacetimidate **6**[12] (724 mg, 1.30 mmol, 1.0 eq.) and Fmoc-Ser-OAll[8] (588 mg, 1.60 mmol, 1.2 eq.) were combined, co-evaporated with toluene (20 ml) and dried under high-vacuum for 2 h. The starting materials were dissolved in Et_2O/CH_2Cl_2 (45 ml, v/v = 2:1) and freshly activated 4 Å molecular sieve was added. The mixture was stirred for 1 h before being cooled to −78 °C. Subsequently the reaction was started by addition $HNTf_2$ (37.0 mg, 0.13 mmol, 0.1 eq.). After complete consumption of the rhamnosyl donor was observed by TLC monitoring, the reaction was neutralized by addition of NEt_3 (200 µl) diluted with CH_2Cl_2 (150 ml) and filtered through a short plug of *Hyflo*. Organic solvents were removed under reduced pressure and the crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1) affording **S3** (740 mg, 1.99 mmol, 76%) as a colourless oil.

R$_f$ = 0.19 (^{c}Hex/EtOAc v/v = 3:1).

Optical rotation: $[\alpha]_D^{24}$ =+ 26.4° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.76 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.65 – 7.60 (m, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.32 (t, $J_{CH,CH}$ = 7.4 Hz, 2H, 2 × CH-Fmoc), 6.01 – 5.86 (m, 2H, H-2´, NH-Fmoc), 5.36 (d, $J_{H3´trans, H2´}$ = 17.2 Hz, 1H, H-3´$_{trans}$), 5.28 – 5.20 (m, 2H, H-3´$_{cis}$, H-4), 4.75 (s, 1H, H-1), 4.70 (d, $J_{H1´,H2´}$ = 5.7 Hz, 2H, H-1´), 4.59 (dd, $J_{Hα,NH}$ = 8.3 Hz, $J_{Hα,Hβ}$ = 3.9 Hz, 1H, H-α), 4.48 – 4.36 (m, 2H, CH_2-Fmoc), 4.28 – 4.22 (m, 3H, CH-Fmoc, H-2, H-3), 4.22 – 4.16 (m, 2H, H-β), 3.57 (dq, $J_{H5,H4}$ = 12.4 Hz, $J_{H5,CH3}$ = 6.4 Hz, 1H, H-5), 1.62 (s, 3H, CH_3, Isopropylidene), 1.38 (s, 3H, CH_3, Isopropylidene), 1.31 (d, $J_{CH3,H5}$ = 6.3 Hz, 3H, rha-CH_3). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ [ppm] = 169.9 (C=O), 158.2 (C=O), 156.3 (Fmoc-C=O), 146.6 – 146.4 (m, CF), 144.9 – 143.9 (m, CF), 144.5 – 144.3 (m, CF),144.0 (Cq-Fmoc), 143.9 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 138.8 –138.6 (m, CF), 137.1–136.9 (m, CF), 131.7 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.3 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 118.8 (C-3´), 111.7 (C_q), 99.1 (C-1), 76.8 (C-3), 76.7 (C-4), 74.3 (C-2), 70.5 (C-β), 70.0 (C-5), 67.4 (CH_2-Fmoc), 66.4 (C-1´), 54.6 (C-α), 47.3 (CH-Fmoc), 27.5 (CH_3, Isopropylidene), 26.3 (CH_3, Isopropylidene), 17.9 (rha-CH_3). [ppm]

Note: Due to low signal intensity, the quaternary C-atom of the pentafluorobenzoyl protecting group could not be assigned in the ^{13}C-NMR spectrum.

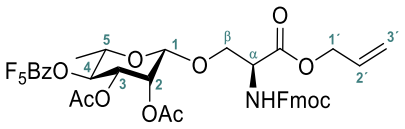
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 160 Hz.

^{19}F-NMR (377 MHz, $CDCl_3$): δ [ppm] = −137.5 (dp, *J* = 17.0 Hz, *J* = 5.8 Hz), −147.5 (tt, *J* = 21.0 Hz, *J* = 4.9 Hz), −159.8 – −160.0 (m).

HRMS (ESI+): Calculated for $C_{37}H_{38}F_5O_{10}N_2$ $[M+NH_4]^+$: 765.2441; found: 765.2446.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 22.23 min, λ = 230 nm.

N^{α}-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-Acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamno-pyranosyl)-L-serine allyl ester (**S5**)



Compound **S3** (980 mg**,** 1.31 mmol, 1.0 eq.) dissolved in 90% aqueous TFA (10 ml) and stirred for 1 h at ambient temperature. After complete conversion of the starting material was observed by TLC monitoring, the reaction was diluted with toluene (20 ml) and concentrated under reduced pressure. The crude residue was co-evaporated with toluene (2 × 15 ml) and dried in high-vacuum for 1 h. The crude diol was dissolved in acetic acid anhydride (15 ml) and pyridine (1 ml) was added carefully. The reaction was stirred at ambient temperature until TLC monitoring indicated complete disappearance of the starting material. The reaction was diluted with EtOAc (35 ml) and washed with 1 M HCl (15 ml), sat. aq. $NaHCO_3$ (3 × 30 ml) and brine

(15 ml) and dried with $MgSO_4$. The crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1) affording **S5** (904 mg, 1.14 mmol, 87% over 2 steps) as a colourless oil.

R_f = 0.41 (^{c}Hex/EtOAc v/v = 2:1).

Optical rotation: $[\alpha]_D^{22}$ = + 46.8° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (800 MHz, $CDCl_3$): δ = 7.77 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.65 – 7.57 (m, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.32 (td, $J_{CH,CH}$ = 7.4 Hz, $J_{CH,CH}$ = 4.2 Hz, 2H, 2 × CH-Fmoc), 5.94 – 5.87 (m, 1H, H-2´), 5.86 (d, $J_{NH,H\alpha}$ = 8.7 Hz, 1H, NH-Fmoc), 5.46 (d, $J_{H2,H3}$ = 3.3 Hz, 1H, H-2), 5.35 – 5.29 (m, 2H, H-4, H-3´$_{trans}$), 5.29 – 5.25 (m, 1H, H-3´$_{cis}$), 5.07 (dd, $J_{H3,H4}$ = 10.1 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 4.70 – 4.61 (m, 3H, H-1, H-1´), 4.52 (dd, $J_{H\alpha,NH}$ = 8.8 Hz, $J_{H\alpha,H\beta}$ = 3.0 Hz, 1H, H-α), 4.47 (dd, J_{CH2} = 10.8 Hz, $J_{CH,CH}$ = 7.0 Hz, 1H, CH_2-Fmoc), 4.38 (dd, $J_{CH,CH}$ = 10.6 Hz, $J_{CH,CH}$ = 7.2 Hz, 1H, CH_2-Fmoc), 4.25 (t, $J_{CH,CH}$ = 7.1 Hz, 1H, CH-Fmoc), 4.19 (dd, $J_{H\beta,H\beta'}$ = 10.4 Hz, $J_{H\beta,H\alpha}$ = 3.1 Hz, 1H, H-β), 4.06 (dd, $J_{H\beta',H\beta}$ = 10.5 Hz, $J_{H\beta',H\alpha}$ = 2.8 Hz, 1H, H-β´), 3.61 (dq, $J_{H5,H4}$ = 12.2 Hz, $J_{H5,CH3}$ = 6.2 Hz, 1H, H-5), 2.19 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.34 (d, $J_{CH3,H5}$ = 6.1 Hz, 3H, rha-CH_3). [ppm]

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.4 (C=O), 170.1 (C=O), 169.4 (C=O), 158.2 (C=O), 156.2 (Fmoc-C=O), 146.4 – 146.2 (m, CF), 144.1 – 143.6 (m, 3C, CF, 2 × Cq-Fmoc), 142.4 – 142.1 (m, CF), 141.5 (Cq-Fmoc), 141.4 (Cq-Fmoc), 139.3 – 138.9 (m, CF), 136.8 –136.4 (m, CF), 131.6 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.3 (CH-Fmoc), 125.2 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc) 119.2 (C-3´), 98.5 (C-1), 73.0 (C-4), 70.6 (2C, C-3, C-5), 70.4 (C-β), 68.8 (C-2), 67.2 (CH_2-Fmoc), 66.5 (C-1´), 54.5 (C-α), 47.3 (CH-Fmoc), 20.9 (OAc), 20.6 (OAc), 17.4 (rha-CH_3). [ppm]

Note: Quaternary C-atom of the pentafluorobenzoyl protecting group could not be assigned in the ^{13}C-NMR spectrum.

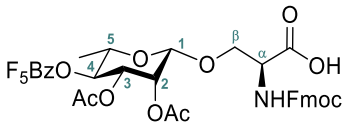
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 159 Hz.

^{19}F-NMR (377 MHz, $CDCl_3$): δ = − 139.0 (dp, *J* = 16.4 Hz, *J* = 5.8 Hz), −147.7 (tt, *J* = 20.8 Hz, *J* = 4.4 Hz), −159.3 – −159.6 (m) [ppm]

HRMS (ESI+): Calculated for $C_{38}H_{38}F_5O_{12}N_2$ $[M+NH_4]^+$: 809.2339; found: 809.2350.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 21.68 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-Acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamno-pyranosyl) -L-serine (**4)**



Cleavage of the allylic ester was conducted according to a literature known protocol.[13]

To a magnetically stirred solution of allylic ester **S5** (700 mg, 0.88 mmol, 1.0 eq.) in dry THF (20 ml), [$Pd(PPh_3)_4$] (104 mg, 0.09 mmol, 0.1 eq.) and *N*-methylaniline (953 µl, 8.80 mmol, 10 eq.) was added. The reaction was stirred at ambient temperature until TLC monitoring indicated complete conversion of the starting material. Subsequently, the reaction was concentrated to dryness under reduced pressure and the crude residue was co-evaporated with toluene (2 × 10 ml). The crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1 + 1% AcOH → 1:1 + 1% AcOH) affording **4** (650 mg, 0.86 mmol, 98%) as a colourless foam.

R*f* = 0.12 (^{c}Hex/EtOAc v/v = 1:1 + 1% AcOH).

Optical rotation: $[\alpha]_D^{24}$ = + 63.0° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.77 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.61 (t, $J_{CH,CH}$ = 8.4 Hz 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.4 Hz, 2H, 2 × CH-Fmoc), 7.34 – 7.30 (m, 2H, 2 × CH-Fmoc), 5.94 (d, $J_{NH,H\alpha}$ = 8.4 Hz, 1H, NH-Fmoc), 5.49 (d, $J_{H2,H3}$ = 3.3 Hz, 1H, H-2), 5.31 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.8 Hz, 1H, H-4), 5.12 (dd, $J_{H3,H4}$ = 10.2 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 4.72 (s, 1H, H-1), 4.55 (d, $J_{H\alpha,NH}$ = 8.2 Hz, 1H, H-α), 4.48 (dd, $J_{CH,CH}$ = 10.6 Hz, $J_{CH,CH}$ = 7.0 Hz, 1H, CH_2-Fmoc), 4.40 (dd, $J_{CH,CH}$ = 10.6 Hz, $J_{CH,CH}$ = 7.1 Hz, 1H, CH_2-Fmoc), 4.27 – 4.16 (m, 2H, CH-Fmoc, H-β), 4.11 (d, $J_{H\beta',H\beta}$ = 10.3 Hz, 1H, H-β´), 3.69 – 3.61 (m, 1H, H-5), 2.19 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.34 (d, $J_{CH3,H5}$ = 6.1 Hz, 3H, rha-CH_3). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 173.2 (C=O), 171.1 (C=O), 170.3 (C=O), 158.2 (C=O), 156.6 (Fmoc-C=O), 146.0 – 145.8 (m, CF), 144.0 – 143.6 (m, 3C, CF, 2 × Cq-Fmoc), 141.5 (Cq-Fmoc) 141.4 (Cq-Fmoc), 138.8 – 138.6 (m, CF), 137.1 – 136.9 (m, CF), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.2 (2C, 2 × CH-Fmoc), 120.2 (2C, 2 × CH-Fmoc), 98.4 (C-1), 73.1 (C-4), 70.7 (C-3), 70.4 (C-5), 69.9 (C-β), 69.2 (C-2), 67.5 (CH_2-Fmoc), 54.2 (C-α), 47.2 (CH-Fmoc), 20.9 (OAc), 20.6 (OAc), 17.4 (rha-CH_3). [ppm]

Note: Due to low signal intensity of C-atoms belonging to the pentafluorobenzoyl protecting group only 33 out of 35 carbons were assigned from the ^{13}C-NMR spectrum.

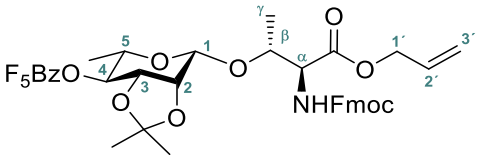
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 159 Hz.

^{19}F-NMR (377 MHz, $CDCl_3$): δ = −139.0 (dp, *J* = 16.5 Hz, *J* = 5.6 Hz), −147.6 (tt, *J* = 21.0 Hz, *J* = 4.4 Hz), −159.3 – −159.5 (m) [ppm].

HRMS (ESI^-): Calculated for $C_{35}H_{29}O_{12}NF_5$ [M-H-]: 750.1615; found: 750.1640.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 19.27 min, λ = 230 nm.

N^{α}-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-Isopropylidene-4-*O*-pentafluorobenzoyl-β-L-rhamno-pyranosyl)-L-threonine allyl ester (**S4**)



2,3-*O*-Isopropylidene-4-*O*-pentafluorobenzoyl-α-L-rhamnosyl trichloroacetimidate **6**[12] (724 mg, 1.33 mmol, 1.0 eq.) and Fmoc-Thr-OAll[8] (610 mg, 1.60 mmol, 1.2 eq.) were combined, co-evaporated with toluene (20 ml) and CH_2Cl_2 (20 ml) and dried under high-vacuum for 2 h. Subsequently the starting materials were dissolved in Et_2O/CH_2Cl_2 (45 ml, v/v = 2:1) and freshly activated 4 Å molecular sieve was added. The mixture was stirred for 1 h before being cooled to − 78 °C. Subsequently the reaction was started by addition $HNTf_2$ (32.0 mg, 0.11 mmol, 0.1 eq.). After complete consumption of the rhamnosyl donor was

observed by TLC monitoring, the reaction was neutralized by addition of NEt_3 (200 µl), diluted with CH_2Cl_2 and filtered through a short plug of *Hyflo*. Organic solvents were removed under reduced pressure and the crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 5:1) affording **S4** (865 mg, 1.14 mmol, 85%) as a colourless foam.

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.80 – 7.74 (m, 2H, 2 × CH-Fmoc), 7.67 – 7.60 (m, 2H, 2 × CH-Fmoc), 7.44 – 7.38 (m, 2H, 2 × CH-Fmoc), 7.32 (td, $J_{CH,CH}$ = 7.2 Hz, $J_{CH,CH}$ = 3.1 Hz, 2H, 2 × CH-Fmoc), 5.93 (ddt, $J_{H2´,H3´trans}$ = 16.4 Hz, $J_{H2´,H3´cis}$ = 10.5 Hz, $J_{H2´,H1´}$ = 5.8 Hz, 1H, H-2´), 5.73 (d, $J_{NH,Hα}$ = 9.6 Hz, 1H, NH-Fmoc), 5.37 (dd, $J_{H3´trans,\ H2´}$ = 17.2 Hz, $J_{H3´trans,\ H1´}$ = 1.5 Hz, 1H, H-3´$_{trans}$), 5.27 (dd, $J_{H3´cis,\ H2´}$ = 10.4 Hz, $J_{H3´cis,\ H1´}$ = 1.3 Hz, 1H, H-3´$_{cis}$), 5.19 (dd, $J_{H4,H5}$ = 9.7 Hz, $J_{H4,H3}$ = 7.4 Hz, 1H, H-4), 4.77 (d, $J_{H1,H2}$ = 2.1 Hz, 1H, H-1), 4.67 (dt, $J_{H1´,H2´}$ =5.9 Hz, $J_{H1´,H3´trans}$ = $J_{H1´,H3´cis}$ = 1.4 Hz, 2H, H-1´), 4.49 (qd, $J_{Hβ,Hγ}$ = 6.4 Hz, $J_{Hβ,Hα}$ = 2.5 Hz, 1H, H-β), 4.45 (dd, $J_{Hα,NH}$ = 9.6 Hz, $J_{Hα,Hβ}$ = 2.5 Hz, 1H, H-α), 4.43 (d, $J_{CH,CH}$ = 7.2 Hz, 2H, CH_2-Fmoc), 4.26 (t, $J_{CH,CH}$ = 7.6 Hz, 1H, CH-Fmoc), 4.19 (dd, $J_{H3,H4}$ = 7.4 Hz, $J_{H3,H2}$ = 5.5 Hz, 1H, H-3), 4.14 (dd, $J_{H2,H3}$ = 5.7 Hz,$J_{H2,H1}$ = 2.2 Hz, 1H, H-2), 3.53 (dq, $J_{H5,H4}$ = 9.8 Hz, $J_{H5,CH3}$ = 6.2 Hz, 1H, H-5), 1.63 (s, 3H, CH_3, Isopropylidene), 1.41 (d, $J_{Hγ,Hβ}$ = 6.5 Hz, 3H, H-γ), 1.38 (s, 3H, CH_3, Isopropylidene), 1.32 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm]

R_f = 0.33 (^{c}Hex/EtOAc v/v = 3:1 + 1% NEt_3).

Optical rotation: $[\alpha]_D^{24}$ = + 21.6° (c = 0.33, $CHCl_3$).

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.3 (C=O), 158.3 (C=O), 156.9 (Fmoc-C=O), 146.6 − 146.4 (m, CF-Ar), 144.9 − 144.7 (m, CF-Ar), 144.0 (Cq-Fmoc), 143.8 (Cq-Fmoc), 142.8 − 142.6 (m, CF-Ar), 141.5 (Cq-Fmoc), 141.4 (Cq-Fmoc), 138.8 − 138.6 (m, CF-Ar), 137.1 − 136.9 (m, CF-Ar), 131.6 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.3 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 119.3 (C-3´), 111.7 (C_q), 99.5 (C-1), 77.0 (C-β), 76.9 (2C,C-3, C-4), 74.6 (C-2), 69.8 (C-5), 67.4 (CH_2-Fmoc), 66.3 (C-1´), 58.8 (C-α), 47.3 (CH-Fmoc), 27.7 (CH_3, Isopropylidene), 26.3 (CH_3, Isopropylidene), 19.0 (Cγ), 17.9 (rha-CH_3). [ppm].

Note: Signal of C-β was obscured by the solvent signal and was therefore assigned by 2D-HSQC analysis. Quaternary C-atom of the pentafluorobenzoyl protecting group could not be assigned in the ^{13}C-NMR spectrum.

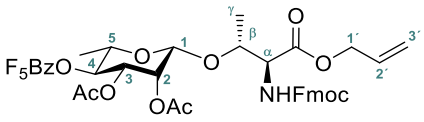
^{19}F-NMR (377 MHz, $CDCl_3$): δ = − 137.5 (dp, *J* = 16.9 Hz, *J* = 5.8 Hz), −147.7 (tt, *J* = 20.8 Hz, *J* = 4.9 Hz), −159.9 – −160.1 (m) [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 159 Hz.

HRMS (ESI+): Calculated for $C_{38}H_{40}F_5O_{10}N_2$ $[M+NH_4]^+$ 779.2598; found: 779.2603.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min):
t_R = 23.57 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-O-Acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamno-pyranosyl) -L-threonine allyl ester (**S6**)



Rhamnosyl amino acid **S4** (590 mg, 0.78 mmol, 1.0 eq.) was dissolved in 90% aqueous TFA (30 ml) and stirred for 1 h at ambient temperature. After TLC monitoring indicated complete conversion of the starting material, TFA was removed under reduced pressure and the crude residue was co-evaporated with toluene (20 ml) and dried 1 h under high vacuum. The crude diol was then dissolved in acetic anhydride (15 ml) and pyridine (1 ml) was added. The reaction was left to stir 1.5 h at room temperature, before being poured onto water. The aqueous mixture carefully neutralized with Na_2CO_3 and subsequently extracted with CH_2Cl_2 (2 × 20 ml). The combined organic layers were washed with brine (25 ml) and dried with $MgSO_4$. The crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1) affording **S6** (463 mg, 0.58 mmol, 74% over 2 steps) as a colourless foam.

R_f = 0.53 (^{c}Hex/EtOAc v/v = 2:1).

Optical rotation: $[\alpha]_D^{24}$ = + 21.0° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.77 (d, $J_{CH,CH}$ = 7.6 Hz, 2H, 2 × CH-Fmoc), 7.67 – 7.61 (m, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.2 Hz, 2H, 2 × CH-Fmoc), 7.32 (t $J_{CH,CH}$ = 7.2 Hz, 2H, 2 × CH-Fmoc), 5.94 (ddt, $J_{H2´,H3´trans}$ = 16.5 Hz, $J_{H2´,H3´cis}$ = 10.4 Hz, $J_{H2´,H1´}$ = 5.9 Hz, 1H, H-2´), 5.60 (d, $J_{NH,Hα}$ = 9.7 Hz, 1H, NH-Fmoc), 5.41 – 5.35 (m, 2H, H-3´$_{trans}$, H-2), 5.33 – 5.27 (m, 2H, H-3´$_{cis}$, H-4), 5.05 (dd, $J_{H3,H4}$ = 10.2 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 4.71 – 4.62 (m, 3H, H-1´, H-

1), 4.49 – 4.35 (m, 4H, CH_2-Fmoc, H-β, H-α), 4.26 (t, $J_{CH,CH}$ = = 7.3 Hz, 1H, CH-Fmoc), 3.62 (dq, $J_{H5,H4}$ = 9.7 Hz, $J_{H5,CH3}$ = 6.3 Hz, 1H, H-5), 2.23 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.39 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3), 1.33 (d, $J_{Hγ,Hβ}$ = 6.4 Hz, 3H, H-γ). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.6 (C=O), 170.2 (C=O), 170.0 (C=O), 158.2 (C=O), 156.8 (Fmoc-C=O), 146.0 – 145.8 (m, CF), 144.4 – 144.2 (m, CF), 144.1 (Cq-Fmoc), 143.8 (Cq-Fmoc), 142.8 – 142.5 (m, CF), 141.5 (Cq-Fmoc), 141.4 (Cq-Fmoc), 138.8 – 138.6 (m, CF), 137.1 – 136.9 (m, CF), 131.5 (C-2´), 127.9 (CH-Fmoc), 127.8 (CH-Fmoc) 127.2 (2C, 2 × CH-Fmoc), 125.4 (CH-Fmoc), 125.3 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 119.8 (C-3´), 98.9 (C-1), 77.7 (C-β), 73.1 (C-4), 70.6 (C-3), 70.3 (C-5), 69.5 (C-2), 67.4 (CH_2-Fmoc), 66.6 (C-1´), 58.7 (C-α), 47.3 (CH-Fmoc), 21.1 (OAc), 20.5 (OAc), 18.3 (C-γ), 17.5 (CH_3-rha). [ppm]

Note: Quaternary C-atom of the pentafluorobenzoyl protecting group could not be assigned in the ^{13}C-NMR spectrum.

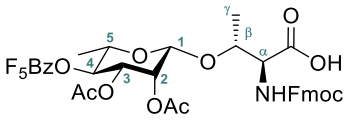
^{19}F-NMR (377 MHz, $CDCl_3$): δ = −138.9 – −139.1 (m), −147.7 (tt, J = 20.8 Hz, J = 4.4 Hz), −159.3 – −159.6 (m) [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 161 Hz.

HRMS (ESI+): Calculated for $C_{39}H_{40}F_5O_{10}N_2$ $[M+NH_4]^+$: 823.2496; found: 823.2498.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 25.88 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-Acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamno-pyranosyl)-L-threonine (**5**)



Cleavage of the allylic ester was conducted according to a literature known protocol.[13]

To a magnetically stirred solution of rhamnosyl threonine derivative **S6** (1.15 g, 1.43 mmol, 1.0 eq.) in dry THF (25 ml), [$Pd(PPh_3)_4$] (162 mg, 0.14 mmol, 0.1 eq.) and *N*-methylaniline (1.55 ml, 14.3 mmol, 10 eq.) was added. The reaction was stirred at ambient temperature until TLC monitoring indicated complete conversion of the starting material. Subsequently, the reaction was concentrated to dryness under reduced pressure and the crude residue was co-evaporated with toluene (2 × 10 ml). The crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 3:1 + 1% AcOH → 2:1 + 1% AcOH) affording **5** (1.03 g, 1.35 mmol, 94%) as a colourless foam.

R$_f$ = 0.33 (^{c}Hex/EtOAc v/v = 1:1 + 1% AcOH).

Optical rotation: $[\alpha]_D^{24}$ = + 37.8 (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.76 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.64 – 7.58 (m, 2H, 2 × CH-Fmoc), 7.42 – 7.37 (m, 2H, 2 × CH-Fmoc), 7.30 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 5.78 (d, $J_{NH,H\alpha}$ = 9.4 Hz, 1H, NH-Fmoc), 5.45 (d, $J_{H2,H3}$ = 3.4 Hz, 1H, H-2), 5.31 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.9 Hz, 1H, H-4), 5.17 (dd, $J_{H3,H4}$ = 10.2 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 4.85 (s, 1H, H-1), 4.52 – 4.45 (m, 2H, H-β, H-α), 4.43 – 4.35 (m, 2H, CH_2-Fmoc), 4.22 (t, $J_{CH,CH}$ = 7.3 Hz, 1H, CH-Fmoc), 3.73 – 3.66 (m, 1H, H-5), 2.24 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.39 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3), 1.34 (d, $J_{H\gamma,H\beta}$ = 6.4 Hz, 3H, H-γ). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 172.9 (C=O), 171.7 (C=O), 170.4 (C=O), 158.2 (C=O), 157.1 (Fmoc-C=O), 145.9 − 145.8 (m, CF-Ar), 144.0 (Cq-Fmoc), 143.7 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 138.8 − 138.5 (m, CF-Ar), 137.1 − 136.9 (m, CF-Ar), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.4 (CH-Fmoc), 125.3 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 98.8 (C-1), 77.6 (C-β), 73.2 (C-4), 70.7 (C-3), 70.2 (C-5), 69.9 (C-2), 67.6 (CH_2-Fmoc), 58.4 (C-α), 47.2 (CH-Fmoc), 21.1 (OAc), 20.6 (OAc), 18.3 (Cγ), 17.5 (rha-CH_3). [ppm]

Note: Due to low signal intensity of the carbons belonging to the pentafluorobenzoyl protecting group only 33 out of 36 carbon atoms were assigned in the ^{13}C-NMR spectrum.

^{19}F-NMR (377 MHz, $CDCl_3$): δ = −139.1 (dp, *J* = 16.6 Hz, *J* = 5.7 Hz), −147.8 (tt, *J* = 20.7 Hz, *J* = 4.3 Hz), −159.5 – −159.7 (m) [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 159 Hz.

HRMS (ESI+): Calculated for $C_{36}H_{36}F_5O_{12}N_2$ $[M+NH_4]^+$: 783. 2183; found: 783.2178.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 21.03 min, λ = 230 nm.

Synthesis of *N*-rhamnosyl asparagine SPPS building blocks:

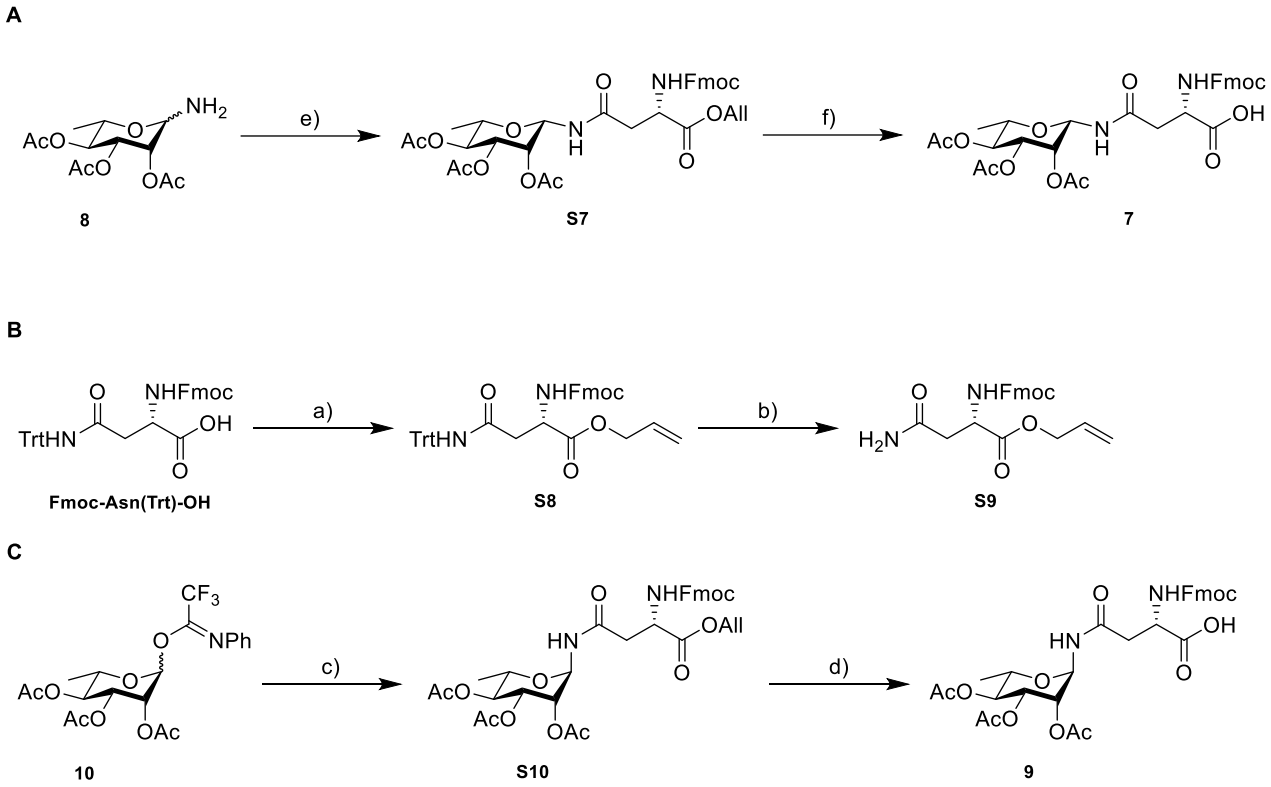
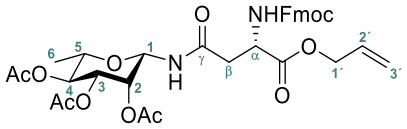


Figure 4: General overview of the chemical synthesis of *N*-rhamnosylated asparagine SPPS building blocks. **A**) Synthesis of β-*N*-rhamnosyl asparagine building block; Reagents and conditions: e) Fmoc-Asp-OAll, PyBOP, DIPEA, DMF, 79%; f) $PhSiH_3$, $[Pd(PPh_3)_4]$, CH_2Cl_2, 81%; **B**) Synthesis of Fmoc-Asn-OAll acceptor **S10**: Reagents and conditions: a) $NaHCO_3$, Allyl bromide, DMF, 85%, b) TFA, H_2O, CH_2Cl_2, 89%. **C**) Synthesis of α-*N*-rhamnosyl asparagine building block: Reagents and conditions: c) Fmoc-Asn-OAll (**S9**), TMSOTf, $MeNO_2/CH_2Cl_2$, 63%; d) $PhSiH_3$, $[Pd(PPh_3)_4]$, CH_2Cl_2, 76%.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-β-L-rhamnosyl)-L-asparagine allyl ester (**S7**)



To a magnetically stirred solution of Fmoc-Asp-OAll[14] (2.40 g, 6.07 mmol, 1.7 eq.) in DMF (20 ml), PyBOP (4.43 g, 8.50 mmol, 2.4 eq.) were added at 0 °C. The solution was adjusted to pH = 9 by careful addition of DIPEA and stirred for 3 min. Subsequently a solution of rhamnosyl

amine **8**[9] (1.04 g, 3.59 mmol, 1.0 eq.) in DMF (20 ml) was added slowly. After complete addition of the amine, the pH was again adjusted to pH = 9 and the reaction was stirred 2 h at room temperature. Subsequently the organic solvents were removed under reduced pressure and the crude product was dissolved in CH_2Cl_2 (150 ml). The organic layer was washed with saturated aqueous $NaHCO_3$ (2 × 50 ml), 1 M HCl (50 ml), H_2O (25 ml) and Brine (25 ml). The organic layer was dried over $MgSO_4$ and all organic solvent were removed under reduced pressure. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH v/v = 120:1) furnishing **S7** (1.89 g, 2.84 mmol, 79%).

R_f = 0.16 (CH_2Cl_2/MeOH v/v = 120:1).

Optical rotation: $[\alpha]_D^{18}$ = + 13.8° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.76 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.60 (t, $J_{CH,CH}$ = 6.6 Hz, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.31 (tt, $J_{CH,CH}$ = 7.4 Hz, $J_{CH,CH}$ = 1.4 Hz, 2H, 2 × CH-Fmoc), 6.49 (d, $J_{NH,H1}$ = 9.2 Hz, 1H, NHγ), 5.90 (m,2H, NH-Fmoc ,H-2´), 5.47 (d, $J_{H1,NH}$ = 9.0 Hz, 1H, H-1), 5.36 – 5.27 (m, 2H, H-2, H-3´trans), 5.24 (d, 1H, $J_{H3´cis,\ H2´}$ = 10.4 Hz, H-3´cis), 5.07 – 4.97 (m, 2H, H-3, H-4), 4.67 (d, $J_{H1´;H2´}$ = 5.8 Hz, H-1´), 4.62 (dt, $J_{Hα,NH}$ = 8.9 Hz, $J_{Hα,Hβ}$ = 4.7 Hz, 1H, H-α), 4.42 – 4.31 (m, 2H, CH_2-Fmoc), 4.23 (t, $J_{CH,CH}$ = 7.0 Hz, 1H, CH-Fmoc), 3.62 (dq, $J_{H5,H4}$ = 8.9 Hz, $J_{H5,CH3}$ = 6.2 Hz, H-5), 2.96 (dd, $J_{Hβ,Hβ´}$ =16.4 Hz, $J_{Hβ,Hα}$ = 4.8 Hz, 1H, H-β), 2.84 (dd, $J_{Hβ´,Hβ}$ = 16.4 Hz, $J_{Hβ´,Hα}$ = 4.5 Hz, 1H, H-β´), 2.21 (s, 3H, OAc), 2.06 (s, 3H, OAc), 1.97 (s, 3H, OAc), 1.23 (d, $J_{CH3,H5}$ = 6.1 Hz, 3H, rha-CH_3). [ppm]

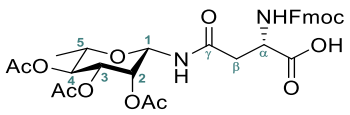
^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.6 (C=O), 170.5 (C=O) 170.1 (2C, 2 × C=O), 169.5 (C=O), 156.3 (C=O-Fmoc), 143.9 (2C, 2 × Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 131.6 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.3 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 119.2 (C-3´), 75.8 (C-1), 72.5 (C-5), 71.6 (C3/C4), 70.2 (2C, C3/C4, C-2), 67.5 (CH_2-Fmoc), 66.7 (C-1´), 50.6 (C-α), 47.2 (CH-Fmoc), 38.3 (C-β), 21.1 (OAc), 20.9 (OAc), 20.7 (OAc), 17.6 (rha-CH_3). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 154 Hz.

HRMS (ESI+): Calculated for $C_{34}H_{39}O_{12}N_2$ $[M+H]^+$: 667.2498; found: 667.2498.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 12.09 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-β-L-rhamnosyl)-L-asparagine (**7**)



Cleavage of the allylic ester was conducted according to a slightly modified procedure.[9]

A magnetically stirred solution of **S7** (1.00 g, 1.50 mmol, 1.0 eq.) in dry CH_2Cl_2 (25 ml) were added Phenylsilane (0.41 ml, 3.30 mmol, 2.2 eq.) and [$Pd(PPh_3)_4$] (35.0 mg, 30.0 µmol, 0.02 eq.). Upon completion, H_2O (20 ml) was added and the reaction was stirred for another 20 min at room temperature. Subsequently, solvents were removed under reduced pressure and the crude residue was co-evaporated with toluene to furnish a black amorphous solid. The crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 1:2 + 1% AcOH) affording **7** (758 mg, 1.21 mmol, 81%) as a beige foam.

R$_f$ = 0.06 (^{c}Hex/EtOAc v/v = 1:2 + 1% AcOH).

Optical rotation: $[\alpha]_D^{18}$ = + 22.6° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.74 (d, $J_{CH,CH}$ = 7.6 Hz, 2H, 2 × CH-Fmoc), 7.61 – 7.57 (m, 2H, 2 × CH-Fmoc), 7.38 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.29 (td, $J_{CH,CH}$ = 7.4 Hz, $J_{CH,CH}$ = 3.3 Hz, 2H, 2 × CH-Fmoc), 7.06 (d, $J_{NH,H1}$ = 9.0 Hz, 1H, NHγ), 6.13 (d, $J_{NH,H\alpha}$ = 7.6 Hz, 1H, NH-Fmoc), 5.49 (dd, $J_{H1,NH}$ = 9.1 Hz, $J_{H1,H2}$ = 1.4 Hz, 1H, H-1), 5.38 (dd, $J_{H2,H3}$ = 3.2 Hz, $J_{H2,H1}$ = 1.2 Hz, 1H, H-2), 5.08 (dd, $J_{H3,H4}$ = 10.2 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 5.01 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.8 Hz, 1H, H-4), 4.62 – 4.56 (m, 1H, H-α), 4.44-4.29 (m, 2H, CH_2-Fmoc), 4.21 (t, $J_{CH,CH}$ = 7.2 Hz, 1H, CH-Fmoc), 3.67 (dq, $J_{H5,H4}$ = 9.5 Hz, $J_{H6,CH3}$ = 6.1 Hz, 1H, H-5), 3.00 – 2.93 (m, 1H, H-β), 2.82 (dd, $J_{H\beta',H\beta}$ = 16.4 Hz, $J_{H\beta',H\alpha}$ = 5.6 Hz, 1H, H-β´), 2.17 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.97 (s, 3H, OAc), 1.22 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3). [ppm].

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 173.2 (C=O) , 170.8 (C=O), 170.2 (2C, 2 × C=O), 156.6 (C=O-Fmoc), 143.8 (2C, 2 × Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.3 (2C, 2 × CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 76.0 (C-1), 72.6 (C-5), 71.6 (C-3), 70.2 (C-4), 70.1 (C-2), 67.7 (CH_2-Fmoc), 50.3 (C-α), 47.1 (CH-Fmoc), 37.9 (C-β), 21.0 (OAc), 20.9 (OAc), 20.7 (OAc), 17.6 (rha-CH_3). [ppm]

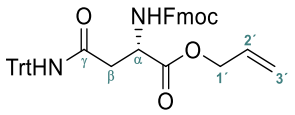
Note: Due to signal overlap only 30 out of 31 carbon atoms are assigned in the ^{13}C-spectra.

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 157 Hz.

HRMS (ESI+): Calculated for $C_{31}H_{38}O_{12}N_3$ [M+NH_4^+]: 644.2450; found: 644.2447.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 18.27 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-trityl-L-asparagine allyl ester (**S8**)



Compound **S8** was synthesized according to a slightly modified procedure.[15]

To a magnetically stirred mixture of Fmoc-Asn(Trt)-OH (3.00 g, 5.03 mmol, 1.0 eq.) and $NaHCO_3$ (1.06 g, 12.6 mmol, 2.5 eq.) in DMF (50 ml), Allyl bromide (1.30 ml, 15.1 mmol, 3.0 eq.) was added slowly. The reaction mixture was stirred 48 h at room temperature. Subsequently, solvents were removed under reduced pressure and the crude residue was dissolved in CH_2Cl_2 (150 ml), washed with water (20 ml) and Brine (20 ml) and dried with $MgSO_4$. Solvent was again removed under reduced pressure and the crude residue was crystallized from ^{c}Hex/EtOAc (v/v = 10:1) to furnish **S8** (2.73 g, 4.29 mmol, 85%) as a colourless solid.

R_f = 0.30 (3:1 ^{c}Hex/EtOAc).

Optical rotation: $[\alpha]_D^{22}$ = + 16.0° (c = 1.0; $CHCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.76 (dd, $J_{CH,CH}$ = 7.6 Hz, $J_{CH,CH}$ = 4.0 Hz, 2H, 2 × CH-Fmoc), 7.59 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.6 Hz, 2H, 2 × CH-Fmoc), 7.34 – 7.27 (m, 10H, 2 × CH-Fmoc, Ar-H), 7.20 – 7.14 (m, 7H, Ar-H), 6.68 (s, 1H, NH_γ), 6.12 (d, $J_{NH,H\alpha}$ = 8.9 Hz, 1H, NH-Fmoc), 5.83 (ddt, $J_{H2´,H3´trans}$ = 16.4 Hz, $J_{H2´,H3´cis}$ = 10.9 Hz, $J_{H2´,H1´}$ = 5.7 Hz, 1H, H-2´), 5.31 – 5.24 (m, 1H, H-3´$_{trans}$), 5.19 (dd, $J_{H3´cis,\ H2´}$ = 10.4 Hz, $J_{H3´cis,\ H3´trans/H1´}$ = 1.4 Hz, 1H, H-3´$_{cis}$), 4.64 (dt, $J_{H\alpha,NH}$ = 8.8 Hz, $J_{H\alpha,H\beta}$ = 4.2 Hz, 1H, H-α), 4.59 (d, $J_{H1´,H2´}$ = 5.7 Hz, 2H, H-1´), 4.42 (dd, $J_{CH,CH}$ = 10.3 Hz, $J_{CH,CH}$ = 7.0 Hz, 1H, CH_2-Fmoc), 4.28 (dd,

$J_{CH,CH}$ = 10.3 Hz, $J_{CH,CH}$ = 7.5 Hz, 1H, CH_2-Fmoc), 4.21 (t, $J_{CH,CH}$ = 7.1 Hz, 1H, CH-Fmoc), 3.14 (dd, $J_{Hβ,Hβ´}$ = 15.9 Hz, $J_{Hβ,Hα}$ = 4.5 Hz, 1H, H-β), 2.83 (dd, $J_{Hβ´,Hβ}$ = 15.9 Hz, $J_{Hβ´,Hα}$ = 4.2 Hz, 1H, H-β´) [ppm]

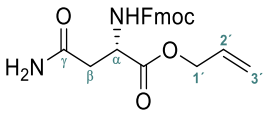
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.8 (C=O), 169.4 (C=O), 156.4 (Fmoc-C=O), 144.4 (C-Ar), 144.0 (Cq-Fmoc), 143.9 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 131.7 (C-2´), 128.8 (C-Ar), 128.2 (C-Ar), 127.8 (2C, 2 × CH-Fmoc), 127.4 (C-Ar), 127.2 (2C, 2 × CH-Fmoc), 125.4 (CH-Fmoc), 125.3 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 118.7 (C-3´), 71.1 (Cq), 67.4 (CH_2-Fmoc), 66.5 (C-1´), 51.1 (C-α), 47.2 (CH-Fmoc), 38.7 (C-β). [ppm]

Note: Due to signal overlap only 27 out of 41 carbon atoms are assigned from the ^{13}C-spectrum.

HRMS (ESI+): Calculated for $C_{41}H_{37}O_5N_2$ $[M+H]^+$: 637.2697; found: 637.2695.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 25.08 min, λ = 230 nm.

N^{α}-Fluorenylmethoxycarbonyl-L-asparagine allyl ester (**S9**)



Compound **S9** was synthesized according to a modified procedure from reference.[16]

To a magnetically stirred solution of asparagine derivative **S8** (2.64 g, 4.15 mmol, 1.0 eq.) in CH_2Cl_2 (20 ml), water (2 ml) and trifluoroacetic acid (20 ml) were added. The reaction mixture was stirred at room temperature until complete conversion of the starting material was observed by TLC monitoring. Subsequently, all organic solvents were removed under reduced pressure and the crude residue was co-evaporated with toluene (2 × 25 ml) and dried under high vacuum. The crude product was then crystallized from cold ^{c}Hex, to furnish **S9** (1.45 g, 3.68 mmol, 89%) as a colourless solid.

R_f = 0.03 (2:1 ^{c}Hex/EtOAc + 1% NEt_3).

Optical rotation: $[\alpha]_D^{23}$ = + 22.0° (c = 0.5; $CHCl_3$).

^{1}H-NMR (400 MHz, DMSO-d_6): δ = 7.89 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.75 (d, $J_{NH,H\alpha}$ = 8.2 Hz, 1H, NH-Fmoc), 7.70 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.42 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.33 (tt, $J_{CH,CH}$ = 7.4 Hz, $J_{CH,CH}$ = 1.5 Hz, 2H, 2 × CH-Fmoc), 5.87 (ddt, $J_{H2',H3'trans}$ = 17.3 Hz, $J_{H2',H3'cis}$ = 10.4 Hz, $J_{H2',H1'}$ = 5.2 Hz, 1H, H-2´), 5.30 (dq, $J_{H3'trans,H2'}$ = 17.3 Hz, $J_{H3'trans,H1'}$ = $J_{H3'trans,H3'cis}$ = 1.8 Hz, 1H, H-3´trans), 5.18 (dq, $J_{H3'cis,H2'}$ = 10.6 Hz, $J_{H3'cis,H1'}$ = $J_{H3'cis,H3'trans}$ = 1.5 Hz, 1H, H-3´cis), 4.58 – 4.53 (m, 2H, H-1´), 4.51 – 4.41 (m, 1H, H-α), 4.34 – 4.17 (m, 3H, CH-Fmoc, CH_2-Fmoc), 2.60 (dd, $J_{H\beta,H\beta}$ = 15.6 Hz, $J_{H\beta,H\alpha}$ = 5.7 Hz, 1H, H-β), 2.54 – 2.45 (m, 1H, H-β´). [ppm]

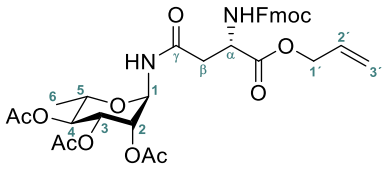
^{13}C-NMR (100 MHz, DMSO-d_6): δ = 171.4 (C=O), 170.8 (C=O), 155.9 (Fmoc-C=O), 143.8 (2C, 2 × Cq-Fmoc), 140.7 (Cq-Fmoc), 132.4 (C-2´), 127.7 (2C, 2 × CH-Fmoc), 127.1 (2C, 2 × CH-Fmoc), 125.3 (2C, 2 × CH-Fmoc), 120.2 (2C, 2 × CH-Fmoc), 117.5 (C-3´), 65.8 (CH_2-Fmoc), 64.9 (C-1´), 50.7 (C-α), 46.6 (CH-Fmoc), 36.7 (C-β). [ppm].

Note: Due to Signal overlapping, only 3 out of the 4 quaternary carbons belonging to the Fmoc-Protecting group can be assigned from the ^{13}C NMR spectrum.

HRMS (ESI+): Calculated for $C_{22}H_{23}O_5N_2$ $[M+H]^+$: 395.1601; found: 395.1601.

RP-HPLC (Luna, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 8.67 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-α-L-rhamnosyl)-L-asparagine allyl ester (**S10**)



Fmoc-Asn-OAll **S9** (1.03 g, 2.60 mmol, 1.0 eq.) and Trifluoroacetimidate **10**[17] (2.40 g, 5.20 mmol, 2.0 eq.) were co-evaporated with dry toluene (2 × 20 ml) and dried in high vacuum for 1 h. The reactants were solved in a mixture of $MeNO_2/CH_2Cl_2$ (v/v = 3:2, 50 ml). Subsequently, freshly activated 4 Å molecular sieve was added and the reaction solution was stirred for 0.5 h at room temperature. After cooling the reaction mixture to 0 °C, TMSOTf (94.0 µl, 0.52 mmol, 0.2 eq.) was added. The reaction was allowed to warm to ambient temperature and was stirred until it was deemed complete. The reaction was stopped by addition of Triethylamine (2 ml) and filtered through a short pad of *Hyflo*®. The filter cake was washed with CH_2Cl_2 (150 ml). The combined organic layers were washed with 1 M HCl (75 ml), saturated aqueous $NaHCO_3$ (75 ml) and brine (50 ml) and dried over $MgSO_4$. Organic solvents were removed under reduced pressure and the crude product was subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 2:1 + 1% AcOH) affording **S10** (1.10 g, 1.64 mmol, 63%) as a colourless foam.

R_f = 0.23 (^{c}Hex/EtOAc v/v = 2:1).

Optical rotation: $[\alpha]_D^{24}$ = − 19.8° (c = 0.33, $CHCl_3$).

^{1}H-NMR: (600 MHz, $CDCl_3$): δ = 7.76 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.60 (dd, $J_{CH,CH}$ = 7.5 Hz, $J_{CH,CH}$ = 4.8 Hz, 2H, 2 × CH-Fmoc), 7.40 (t, $J_{CH,CH}$ = 7.4 Hz, 2H, 2 × CH-Fmoc), 7.31 (t, $J_{CH,CH}$ = 7.4 Hz, 2H, 2 × CH-Fmoc), 6.93 (d, $J_{NH,H1}$ = 8.6 Hz, 1H, NHγ), 6.11 (d, $J_{NH,H\alpha}$ = 8.6 Hz, 1H, NH-Fmoc), 5.89 (ddt, $J_{H2´,H3´trans}$ = 16.4 Hz, $J_{H2´,H3´cis}$ = 10.9 Hz, $J_{H2´,H1´}$ = 5.5 Hz, 1H, H-2´), 5.61 (dd, $J_{H1,NH}$ = 8.6 Hz, $J_{H1,H2}$ = 4.1 Hz, 1H, H-1), 5.32 (d, $J_{H3´trans,\ H2´}$ = 17.1 Hz, 1H, H-3´$_{trans}$), 5.27 – 5.18 (m, 3H, H-3´$_{cis}$, H-2, H-3), 4.97 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 7.2 Hz, 1H, H-4), 4.73 – 4.61 (m, 3H, H-α, H-1´), 4.45 (dd, $J_{CH,CH}$ = 10.3 Hz, $J_{CH,CH}$ = 7.0 Hz, 1H, CH_2-Fmoc), 4.30 (dd, $J_{CH,CH}$ = 10.4 Hz, $J_{CH,CH}$ = 7.6 Hz, 1H, CH_2-Fmoc), 4.24 (t, $J_{CH,CH}$ = 7.0 Hz, 1H, CH-Fmoc), 3.86 – 3.79 (m, 1H, H-5), 2.99 (dd, $J_{Hβ,Hβ´}$ = 16.5 Hz, $J_{Hβ,Hα}$ = 5.2 Hz, 1H, H-β), 2.83 (dd, $J_{Hβ´,Hβ}$ =

16.5 Hz, $J_{H\beta',H\alpha}$ = 4.3 Hz, 1H, H-β´), 2.12 (s, 3H, OAc), 2.05 (s, 3H, OAc), 1.97 (s, 3H, OAc), 1.27 (d, $J_{CH3,H5}$ = 6.6 Hz, 3H, rha-CH_3). [ppm]

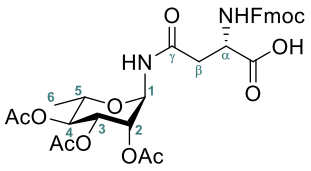
^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.7 (C=O), 170.5 (C=O), 170.3 (C=O), 170.2 (C=O), 169.8(C=O), 156.5 (Fmoc-C=O), 144.0 (Cq-Fmoc), 143.7 (Cq-Fmoc), 141.4 (2C, 2 × Cq-Fmoc), 131.5 (C-2´), 127.9 (2C, 2 × CH-Fmoc), 127.3 (2C, 2 × CH-Fmoc), 125.4 (CH-Fmoc), 125.3 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 119.0 (C-3´), 74.4 (C-1), 71.1 (C-4), 69.7 (C-5), 69.1 (C-2/C-3), 68.7 (C-2/C-3), 67.7 (CH_2-Fmoc), 66.6 (C-1´), 50.8 (C-α), 47.2 (CH-Fmoc), 38.3 (C-β), 20.9 (2C, 2 × OAc), 20.8 (OAc), 17.0 (rha-CH_3). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 169 Hz.

HRMS (ESI+): Calculated for $C_{34}H_{38}O_{12}N_2Na^+$ $[M+Na]^+$: 689.2317; found: 689.2313.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 12.17 min, λ = 230 nm.

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-α-L-rhamnosyl)-L-asparagine (**9**)



Cleavage of the allylic ester was conducted according to a slightly modified procedure.[9]

To a magnetically stirred solution of **S10** (800 mg, 1.35 mmol, 1.0 eq.) in dry CH_2Cl_2 (15 ml), Phenylsilane (328 µl, 2.97 mmol, 2.2 eq.) and $[Pd(PPh_3)_4]$ (32.0 mg, 27.7 µmol, 0.02 eq.) were added. The reaction was stirred at ambient temperature until complete conversion of the starting material was observed *via* TLC monitoring. Subsequently, the reaction was stopped by addition of 1 ml water and solvents were removed under reduced pressure. The crude product was co-evaporated with toluene (2 × 20 ml) and subjected to flash column chromatography (^{c}Hex/EtOAc v/v = 1:1 + 1% AcOH). α-Rhamnosyl asparagine building block **9** (640 mg, 1.02 mmol, 76%) was obtained as a colourless foam.

R_f = 0.10 (^{c}Hex/EtOAc v/v = 1:2 + 1% AcOH).

Optical rotation $[\alpha]_D^{22}$ = − 10.2° (c = 0.33, $CHCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.98 (d, $J_{NH,H1}$ = 8.5 Hz, 1H, NHγ), 7.72 (d, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 7.57 (dd, $J_{CH,CH}$ = 7.6 Hz, $J_{CH,CH}$ = 4.4 Hz, 2H, 2 × CH-Fmoc), 7.36 (t, $J_{CH,CH}$ = 7.4 Hz, 2H, 2 × CH-Fmoc), 7.27 (t, $J_{CH,CH}$ = 7.5 Hz, 2H, 2 × CH-Fmoc), 6.48 (d, $J_{NH,H\alpha}$ = 8.1 Hz, 1H, NH-Fmoc), 5.63 (dd, $J_{H1,NH}$ = 8.6, $J_{H1,H2}$ = 2.6 Hz, 1H, H-1), 5.33 (dd, $J_{H3,H4}$ = 9.3 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 5.26 (t, $J_{H2,H3}$ = $J_{H2,H1}$ = 3.1 Hz, 1H, H-2), 5.03 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.0 Hz, 1H, H-4), 4.69 (q, $J_{H\alpha,NH}$ = $J_{H\alpha,H\beta}$ = 6.0 Hz, 1H, H-α), 4.36 (dd, $J_{CH,CH}$ = 10.5 Hz, $J_{CH,CH}$ = 7.4 Hz, 1H, CH_2-Fmoc), 4.31 (dd, $J_{CH,CH}$ = 10.5 Hz, $J_{CH,CH}$ = 7.4 Hz, 1H, CH_2-Fmoc), 4.18 (t, J = 7.4 Hz, 1H, CH-Fmoc), 3.84 – 3.77 (m, 1H, H-5), 3.04 (dd, $J_{H\beta,H\beta'}$ = 16.3 Hz, $J_{H\beta,H\alpha}$ = 5.6 Hz, 1H, H-β), 2.85 (dd, $J_{H\beta',H\beta}$ = 16.2 Hz, $J_{H\beta',H\alpha}$ = 5.0 Hz, 1H, H-β´), 2.13 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.94 (s, 3H, OAc), 1.20 (d, $J_{CH3,H5}$ = 6.3 Hz, 3H, rha-CH_3). [ppm]

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 173.8 (C=O), 171.5 (C=O), 171.3 (C=O), 170.6 (C=O), 169.9 (C=O), 156.8 (Fmoc-C=O), 143.9 (Cq-Fmoc), 143.7 (Cq-Fmoc), 141.4 (Cq-Fmoc), 141.3 (Cq-Fmoc), 127.9 (2C, 2 × CH-Fmoc), 127.2 (2C, 2 × CH-Fmoc), 125.4 (CH-Fmoc), 125.3 (CH-Fmoc), 120.1 (2C, 2 × CH-Fmoc), 75.7 (C-1), 71.0 (C-4), 69.5 (C-3), 69.3 (C-2), 68.4 (C-5), 67.8 (CH_2-Fmoc), 50.7 (C-α), 47.0 (CH-Fmoc), 37.5 (C-β), 21.0 (2C, 2 × OAc), 20.8 (OAc), 17.4 (rha-CH_3). [ppm]

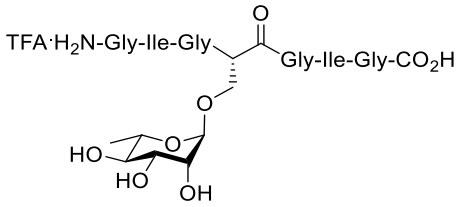
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 168 Hz.

HRMS (ESI+): Calculated for $C_{31}H_{35}O_{12}N_2$ $[M+H^+]^+$: 627.2185, found: 627.2182.

RP-HPLC (Aeris, 0.1% TFA; 0 min 50% B → 30 min 100 % B, flow: 1 ml/min): t_R = 7.28 min, λ = 230 nm.

Synthesis and characterization of rhamnosylated glycopeptide haptens for immunization

Ser$^{\alpha\text{-Rha}}$-Peptide (**11**)



Glycopeptide was synthesized according to general procedure for 0.1 mmol scale peptide synthesis. α-rhamnosyl serine building block **1** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 2 h per coupling. Cleavage of the *O*-acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. Purification *via* RP-HPLC (Gradient: B; λ = 212 nm) yielded **11** (52 mg, 63 µmol, 63%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.45 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_I), 8.35 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.33 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.24 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.12 (d, $J_{NH,H\alpha}$ = 8.1 Hz, 1H, NH_S), 7.97 (bs, 3H, NH_3^+), 7.88 (d, $J_{NH,H\alpha}$ = 8.9 Hz, 1H, NH_I), 4.54 – 4.50 (m, 2H, H-1, $H_{S\alpha}$), 4.31 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.2 Hz, 1H, $H_{I\alpha}$), 4.21 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.1 Hz, 1H, $H_{I\alpha}$), 3.86 – 3.67 (m, 7H, 6 × $H_{G\alpha}$, $H_{S\beta}$), 3.63 – 3.59 (m, 3H, $H_{G\alpha}$, H-2), 3.49 (dd, $J_{H\beta,H\beta}$ = 10.2, $J_{H\beta,H\alpha}$ = 4.6 Hz, 1H, $H_{S\beta}$), 3.40 – 3.33 (m, 2H, H-5, H-3), 3.16 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.4 Hz, 1H, H-4), 1.75 – 1.69 (m, 2H, 2 × $H_{I\beta}$), 1.49 – 1.39 (m, 2H, $H_{I\gamma1}$), 1.11 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3), 1.11 – 1.02 (m, 2H, $H_{I\gamma1}$), 0.86 (d, $J_{H\gamma,H\beta}$ = 6.8 Hz, 3H, $H_{I\gamma2}$), 0.85 (d, $J_{H\gamma,H\beta}$ = 6.8 Hz, 3H, $H_{I\gamma2}$), 0.84 – 0.79 (m, 6H, 2 × $H_{I\delta}$). [ppm]

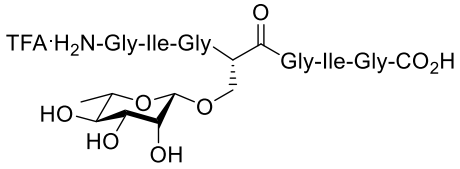
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (C=O), 171.1 (C=O), 170.8 (C=O), 169.4 (C=O), 168.6 (C=O), 168.3 (C=O), 165.8 (C=O), 99.6 (C-1), 71.9 (C-4), 70.6 (C-5/C-3), 70.2 (C-2), 68.5 (C-5/C-3), 66.4 (S_β), 56.9 (I_α), 56.7 (I_α), 52.4 (S_α), 42.0 (G_α), 41.7 (G_α), 40.6 (G_α), 40.2 (G_α), 37.0 (I_β), 36.9 (I_β), 24.2 (2C, 2 × $I_{\gamma1}$), 17.9 (CH_3-rha), 15.3 (2C, 2 × $I_{\gamma2}$), 11.2 (I_δ), 11.1 (I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 169 Hz.

HRMS (ESI+): Calculated for $C_{29}H_{52}N_7O_{13}^+$ $[M+H]^+$: 706.3618; found: 706.3611.

RP-HPLC: (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min**):** t_R = 10.96 min, λ = 230 nm.

Ser$^{\beta\text{-Rha}}$-Peptide (**12**)



Glycopeptide was synthesized according to general procedure for 0.05 mmol scale peptide synthesis. β-Rhamnosyl serine building block (**4**) was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Cleavage of the *O*-ester protecting groups was performed according to the general procedure for on resin-deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. Purification *via* RP-HPLC (Gradient: A; λ = 205 nm) yielded **12** (22 mg, 27 µmol, 54%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.48 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_I), 8.37 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.32 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.23 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.10 (d, $J_{NH,H\alpha}$ = 7.7 Hz, 1H, NH_S), 7.81 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 4.44 – 4.40 (m, 2H, $H_{S\alpha}$, H-1), 4.30 (t, $J_{H\alpha,H\beta}$ = $J_{H\alpha,NH}$ = 8.0 Hz, 1H, $H_{I\alpha}$), 4.22 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 7.1 Hz, 1H, $H_{I\alpha}$), 3.86 – 3.64 (m, 9H, 6 × $H_{G\alpha}$, 2 ×$H_{S\beta}$, H-2), 3.60 (d, $J_{H\alpha,NH}$ = 4.0 Hz, 2H, $H_{G\alpha}$), 3.20 (dd, $J_{H3,H4}$ = 8.8 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 3.13 – 3.04 (m, 2H, H-4, H-5), 1.75 – 1.68 (m, 2H, 2 × $H_{I\beta}$), 1.48 – 1.40 (m, 2H, $H_{I\gamma1}$), 1.17 (d, $J_{CH3,H5}$ = 5.7 Hz, 3H, rha-CH_3), 1.12 – 1.03 (m, 2H, $H_{I\gamma1}$), 0.87 – 0.79 (m, 12H, 2 × $H_{I\gamma2}$, 2 × $H_{I\delta}$). [ppm]

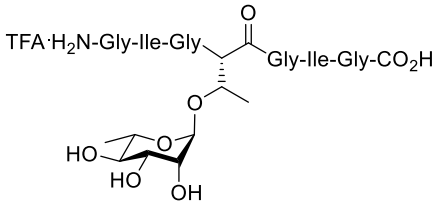
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (C=O), 171.1 (C=O), 170.9 (C=O), 169.7 (C=O), 168.7 (C=O), 168.4 (C=O), 165.9 (C=O), 100.2 (C-1), 73.2 (C-3), 72.2 (C-4/C-5), 72.0 (C-4/C-5), 70.4 (C-2), 68.1 (S_β), 56.9 (I_α), 56.6 (I_α), 53.0 (S_α), 42.0 (G_α), 41.7 (G_α), 40.7 (G_α), 40.2 (G_α), 36.9 (2C, 2 × I_β), 24.2 ($I_{\gamma1}$), 24.1 ($I_{\gamma1}$), 17.9 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (2C, 2 × I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 156 Hz.

HRMS (ESI−): Calculated for $C_{29}H_{50}N_7O_{13}^-$ [M–H]$^-$: 704.3472; found: 704.34718.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min**):** t_R = 11.15 min, λ = 230 nm.

Thr$^{\alpha\text{-Rha}}$-Peptide (**13**)



Glycopeptide **13** was synthesized according to general procedure for 0.1 mmol scale peptide synthesis. α-Rhamnosyl threonine building block **2** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Cleavage of the *O*-acetyl protecting groups were performed according to the general procedure for *on-resin* deprotection. Cleavage from resin according to the general procedure and subsequent purification *via* RP-HPLC purification (Gradient: B; λ = 212 nm) yielded **13** (37 mg, 44 µmol, 44%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.46 (d, $J_{NH,H\alpha}$ = 8.9 Hz, 1H, NH_I), 8.38 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.32 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.07 (t, $J_{NH,H\alpha}$ = 5.6 Hz, 1H, NH_G), 8.00 – 7.93 (m, 4H, NH_I, NH_3^+), 7.79 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_T), 4.59 (d, $J_{H1,H2}$ = 1.6 Hz, 1H, H-1), 4.44 (dd, $J_{H\alpha,NH}$ = 9.1 Hz, $J_{H\alpha,H\beta}$ = 3.7 Hz, 1H, $H_{T\alpha}$), 4.31 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.3 Hz, 1H, $H_{I\alpha}$), 4.21 (dd, $J_{H\alpha,NH}$ = 8.9, $J_{H\alpha,H\beta}$ = 7.0 Hz, 1H, $H_{I\alpha}$), 4.06 (qd, $J_{H\beta,H\gamma}$ = 6.3 Hz, $J_{H\beta,H\alpha}$ = 3.6 Hz, 1H, $H_{T\beta}$), 3.88 – 3.75 (m, 5H, 5× $H_{G\alpha}$), 3.69 (dd, $J_{H\alpha,H\alpha}$ = 17.4 Hz, $J_{H\alpha,NH}$ = 5.8 Hz, $H_{G\alpha}$) 3.63 – 3.58 (m, 2H, 2× $H_{G\alpha}$), 3.56 (dd, $J_{H2,H3}$ = 3.4 Hz, $J_{H2,H1}$ =1.6 Hz, 1H, H-2), 3.40 (dd, $J_{H3,H4}$ = 9.4 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 3.33 (dq, $J_{H5,H4}$ = 9.4 Hz, $J_{H5,CH3}$ = 6.2 Hz, 1H, H-5), 3.15 (t, $J_{H4,H3}$ = $J_{H4,H3}$ = 9.4 Hz, 1H, H-4), 1.75 – 1.69 (m, 2H, 2 × $H_{I\beta}$), 1.49 – 1.40 (m, 2H, $H_{I\gamma1}$), 1.12 – 1.05 (m, 5H, rha-CH_3, $H_{I\gamma1}$), 1.02 (d, $J_{H\gamma,H\beta}$ = 6.2 Hz, 3H, $H_{T\gamma}$), 0.86 (d, $J_{H\gamma,H\beta}$ = 6.8 Hz, 3H, $H_{I\gamma2}$), 0.85 (d, $J_{H\gamma,H\beta}$ = 6.8 Hz, 3H, $H_{I\gamma2}$), 0.82 (t, $J_{H\delta,H\gamma}$ = 7.4 Hz, 3H, $H_{I\delta}$), 0.80 (t, $J_{H\delta,H\gamma}$ = 7.4 Hz, 3H, $H_{I\delta}$). [ppm]

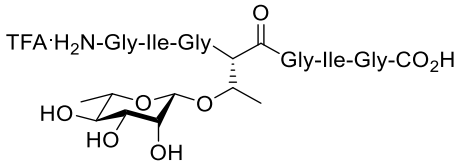
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (C=O), 171.1 (C=O), 170.8 (C=O), 169.5 (C=O), 168.9 (C=O), 168.3 (C=O), 165.8 (C=O), 96.0 (C-1), 71.9 (C-4), 70.7 (C-2), 70.5 (C-3), 69.7 (T_β), 68.6 (C-5), 56.9 (I_α), 56.7 (I_α), 56.6 (T_α), 41.9 (G_α), 40.6 (G_α), 40.2 (G_α), 40.0 (G_α), 37.0 (I_β), 36.8 (I_β), 24.2 (2C, 2 × $I_{\gamma1}$), 17.8 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 14.5 (T_γ), 11.2 (I_δ), 11.1 (I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 169 Hz.

HRMS (ESI+): Calculated for $C_{30}H_{54}N_7O_{13}^+$ $[M+H]^+$: 720.3774; found: 720.3776.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min**):** t_R = 11.10 min, λ = 230 nm.

Thr$^{\beta\text{-Rha}}$-Peptide (**14)**



Glycopeptide was synthesized according to general procedure for 0.05 mmol scale peptide synthesis. β-Rhamnosyl threonine building block **5** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Cleavage of the *O*-ester protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) yielded **14** (18 mg, 22 µmol, 44%) after lyophilization.

^{1}H-NMR: (800 MHz, DMSO-d_6): δ = 8.46 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_I), 8.38 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.33 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.17 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 7.95 (t, $J_{NH,H\alpha}$ = 5.6 Hz, 3H, NH_3^+), 7.92 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 7.82 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_T), 4.45 (s, 1H, H-1), 4.35 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 3.6 Hz, 1H, $H_{T\alpha}$), 4.31 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.2 Hz, 1H, $H_{I\alpha}$), 4.23 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 7.0 Hz, 1H, $H_{I\alpha}$), 4.10 (qd, $J_{H\beta,H\gamma}$ = 6.4 Hz, $J_{H\beta,H\alpha}$ = 3.7 Hz, 1H, $H_{T\beta}$), 3.88 – 3.68 (m, 6H, 6 × $H_{G\alpha}$), 3.63 (d, $J_{H2,H3}$ = 3.4 Hz, 1H, H-2), 3.62 – 3.58 (m, 2H, $H_{G\alpha}$), 3.17 (dd, $J_{H3,H4}$ = 9.0 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 3.10 – 3.01 (m, 2H, H-

4, H-5), 1.76 – 1.69 (m, 2H, 2 × $H_{Iβ}$), 1.50 – 1.39 (m, 2H, $H_{Iγ1}$), 1.15 (d, $J_{CH3,H5}$ = 5.9 Hz, 3H, rha-CH_3), 1.11 (d, $J_{Hγ,\ Hβ}$ = 6.3 Hz, 3H, $H_{Tγ}$), 1.10 – 1.03 (m, 2H, $H_{Iγ1}$), 0.88 – 0.79 (m, 12H, 2 × $H_{Iγ2}$, 2 × $H_{Iδ}$). [ppm]

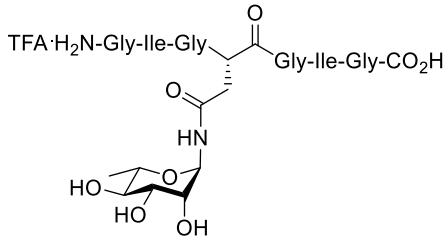
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (C=O), 171.1 (C=O), 170.8 (C=O), 169.8 (C=O), 168.8 (C=O), 168.4 (C=O), 165.8 (C=O), 100.8 (C-1), 75.3 ($T_β$), 73.1 (C-3), 72.0 (C-4/C-5), 71.9 (C-4/C-5), 70.6 (C-2), 56.9 ($I_α$), 56.7 ($I_α$), 56.6 ($T_α$), 41.8 (2C, 2 × $G_α$), 40.6 ($G_α$), 40.2 ($G_α$), 36.9 (2C, 2 × $I_β$), 24.2 ($I_{γ1}$), 24.1 ($I_{γ1}$), 18.8 ($T_γ$), 18.0 (rha-CH_3), 15.3 (2C, 2 × $I_{γ2}$), 11.2 ($I_δ$), 11.1 ($I_δ$). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 156 Hz.

HRMS (ESI−): Calculated for $C_{30}H_{52}N_7O_{13}^-$ [M–H]$^-$: 718.3629; found: 718.3630.

RP-HPLC: (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min**):** t_R = 11.63 min, λ = 230 nm.

Asn$^{α\text{-Rha}}$-Peptide (**15)**



The synthesis of α-rhamnosyl asparagine glycopeptide **15** was performed according to the general procedure for peptide synthesis on a 0.1 mmol scale. α-Rhamnosyl asparagine building block **9** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 2 h per coupling. Deprotection of the acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the general procedure furnished the crude glycopeptide which was subjected to RP-HPLC purification (Gradient: A; λ = 205 nm) to afford **15** (52 mg, 61 µmol, 61%) after lyophilization.

^{1}H-NMR (600 MHz, DMSO-D_6): δ = 8.55 (d, $J_{NH,H\alpha}$ = 8.7 Hz, 1H, NH_I), 8.51 (d, $J_{NH,H1}$ = 8.8 Hz, 1H, $NH_{N\gamma}$), 8.39 (t, $J_{NH,H\alpha}$ = 5.6 Hz, 1H, -NH_G), 8.22 (t, $J_{NH,h\alpha}$ = 5.8 Hz, 1H, NH_G), 8.15 (d, $J_{NH,N\alpha}$ = 7.8 Hz, 1H, NH_N), 8.09 (t, $J_{NH,G\alpha}$ = 5.8 Hz, 1H, NH_G), 7.78 (d, $J_{NH,I\alpha}$ = 9.0 Hz, 1H, NH_I), 5.22 (dd, $J_{H1,NH}$ = 8.8 Hz, $J_{H1,H2}$ = 2.0 Hz, 1H, H-1), 4.55 (td, $J_{N\alpha,NH}$ = 7.8 Hz, $J_{N\alpha,N\beta}$ =5.3 Hz, 1H, $H_{N\alpha}$), 4.29 (dd, $J_{I\alpha,NH}$ = 8.7 Hz, $J_{I\alpha,I\beta}$ = 7.3 Hz, 1H, $H_{I\alpha}$), 4.20 (dd, $J_{I\alpha,NH}$ = 9.0, $J_{I\alpha,I\beta}$ = 7.3 Hz, 1H, $H_{I\alpha}$), 3.83 (dd, $J_{H\alpha,H\alpha}$ = 16.6 Hz, $J_{H\alpha,NH}$ = 6.0 Hz, 1H, $H_{G\alpha}$), 3.77 – 3.67 (m, 5H, $H_{G\alpha}$), 3.64 – 3.60 (m, 3H, $H_{G\alpha}$, H-3), 3.51 (m, 1H, H-2), 3.39 – 3.36 (m, 1H, H-5), 3.20 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.0 Hz, 1H, H-4), 2.60 – 2.55 (m, 2H, 2 × $H_{N\beta}$), 1.76-1.70 (m, 2H, 2 × $H_{I\beta}$), 1.47-1.38 (m, 2H, $H_{I\gamma1}$), 1.11 – 1.04 (m, 5H, rha-CH_3, $H_{I\gamma1}$), 0.86 – 0.78 (m, 12H, 2 × $H_{I\gamma2}$, 2 × $H_{I\delta}$). [ppm]

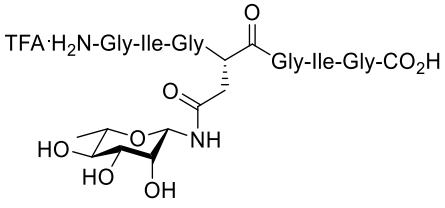
^{13}C-NMR (150 MHz, DMSO-D_6): δ = 171.2 (3C, 3 × C=O), 170.9 (C=O), 169.6 (C=O), 168.6 (C=O), 168.4 (C=O), 166.0 (C=O), 77.8 (C-1), 72.1 (C-4), 70.6 (C-2), 70.2 (C-3), 69.5 (C-5), 57.0 (I_α), 56.7 (I_α), 49.6 (N_α), 42.1 (G_α), 41.8(G_α), 40.8 (G_α),40.2 (G_α) 37.3 (N_β), 36.9 (I_β), 36.6 (I_β), 24.2 (2C, 2 × $I_{\gamma1}$), 18.0 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$),11.1 (I_δ), 11.0 (I_δ). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 165 Hz.

HRMS (ESI+): Calculated for $C_{30}H_{53}O_{13}N_8$ $[M+H]^+$: 733.3727; found: 733.3720.

RP-HPLC (Aeris, 0.1% TFA, 0 min: 8% B → 10 min: 8% B → 60 min: 50% B → 70 min: 100% B; flow: 1 ml/ min): t_R = 22.45 min, λ = 230 nm.

Asn$^{\beta\text{-Rha}}$-Peptide (**16**)



The synthesis of β-rhamnosyl asparagine glycopeptide **16** was performed according to the general procedure for peptide synthesis on a 0.1 mmol scale. β-Rhamnosyl asparagine building block **7** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 2 h per coupling. Deprotection of the acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the general procedure furnished the crude glycopeptide which was

subjected to RP-HPLC purification (Gradient: A; λ = 205 nm) to afford **16** (54 mg, 64 µmol, 64%) after lyophilization.

^{1}H-NMR (600 MHz, DMSO-d_6) δ = 8.53 (d, $J_{NH,H\alpha}$ = 8.6 Hz, 1H, NH_I), 8.37 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.26 – 8.19 (m, 2H, $NH_{N\gamma}$, NH_G), 8.14 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.11 (d, $J_{NH,H\alpha}$ = 8.0 Hz, 1H, NH_N), 7.80 (d, $J_{NH,\,I\alpha}$ = 8.9 Hz, 1H, NH_I), 4.98 (dd, $J_{H1,NH}$ = 9.1 Hz, $J_{H1,H2}$ = 1.3 Hz, 1H, H-1), 4.59 – 4.54 (m, 1H, $H_{N\alpha}$), 4.29 (dd, $J_{H\alpha,NH}$ = 8.7 Hz, $J_{H\alpha,H\beta}$ = 7.3 Hz, 1H, $H_{I\alpha}$), 4.20 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 7.4 Hz, 1H, $H_{I\alpha}$), 3.83 (dd, $J_{H\alpha1,H\alpha2}$ = 16.6 Hz, $J_{H\alpha,NH}$ = 5.9 Hz, 1H, $H_{G\alpha}$), 3.77 (dd, $J_{H\alpha,H\alpha}$ = 17.4 Hz, $J_{H\alpha,NH}$ = 5.9 Hz, 1H, $H_{G\alpha}$), 3.74 – 3.67 (m, 4H, 4 × $H_{G\alpha}$), 3.61 (d, 2H, 2 × $H_{G\alpha}$), 3.56 (d, $J_{H2,H3}$ = 3.2 Hz, 1H, H-2), 3.28 (dd, $J_{H3,H4}$ = 8.6 Hz, $J_{H3,H2}$ = 3.1 Hz, 1H, H-3), 3.13-3.06 (m, 2H, H-4, H-5), 2.65 (dd, $J_{H\beta,H\beta}$ = 15.8 Hz, $J_{H\beta,H\alpha}$ = 5.7 Hz, 1H, $H_{N\beta}$), 2.56-2.51 (m, 1H, $H_{N\beta}$), 1.78 – 1.69 (m, 2H, 2 × $H_{I\beta2}$), 1.49 – 1.39 (m, 2H, $H_{I\gamma1}$), 1.12 – 1.05 (m, 5H, rha-CH_3, $H_{I\gamma1}$), 0.86 – 0.79 (m, 12H, 2 × $I_{\gamma2}$, 2 × I_δ). [ppm]

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 171.2 (2C, 2 × C=O), 171.1 (C=O), 170.8 (C=O), 169.3 (C=O), 168.5 (2C, 2 × C=O), 165.9 (C=O), 77.4 (C-1), 73.7 (C-3), 73.6 (C-4/C-5), 71.6 (C-4/C-5), 70.7 (C-2), 57.0 (I_α), 56.7 (I_α), 49.5 (N_α), 42.2 (G_α), 41.7 (G_α), 40.6 (G_α), 40.2 (G_α), 37.5 (N_β), 36.9 (I_β), 36.5 (I_β), 24.2 (2C, 2 × $I_{\gamma1}$) , 18.0 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (I_δ), 11.0 (I_δ). [ppm]

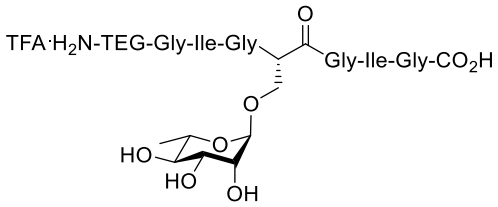
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 154 Hz.

HRMS (ESI+): Calculated for $C_{30}H_{53}O_{13}N_8$ $[M+H]^+$: 733.3727; found: 733.3717.

RP-HPLC (Aeris, 0.1% TFA, 0 min: 5% B → 40 min: 80% B → 60 min: 100% B, flow: 1 ml/ min): t_R = 10.73 min, λ = 230 nm.

Synthesis of BSA-conjugates for Western Blot Analysis

Ser$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer (**S11**)



Glycopeptide was synthesized according to general procedure for 0.1 mmol scale peptide synthesis. α-Rhamnosyl serine building block **1** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Fmoc-TEG-CO_2H Spacer was coupled with a 4-fold excess and a coupling time of 1 h per coupling. Cleavage of the *O*-acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) yielded **S11** (58 mg, 57 µmol, 57%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.33 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.24 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.22 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.11 – 8.08 (m, 2H, NH_G, NH_S), 7.86 (d, $J_{NH,H\alpha}$ = 8.9 Hz, 1H, NH_I), 7.85 (d, $J_{NH,H\alpha}$ = 8.7 Hz, 1H, NH_I), 7.77 (s, 3H, NH_3^+), 4.53 (d, $J_{H1,\ H2}$ = 1.6 Hz, 1H, H-1), 4.51 (ddd, $J_{H\alpha,NH}$ = 8.1 Hz, $J_{H\alpha,H\beta}$ = 6.2 Hz, $J_{H\alpha,H\beta}$ = 4.8 Hz, 1H, $H_{S\alpha}$), 4.24 – 4.17 (m, 2H, 2 × $H_{I\alpha}$), 3.81 – 3.73 (m, 7H, 7 × $H_{G\alpha}$), 3.73 – 3.67 (m, 2H, $H_{G\alpha}$, $H_{S\beta}$), 3.62 – 3.47 (m, 14H, 12 × CH_2-O-Spacer, H-2, $H_{S\beta}$), 3.39 – 3.34 (m, 2H, H-3, H-5), 3.16 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.4 Hz, 1H, H-4), 3.01 – 2.95 (m, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.76 – 1.67 (m, 2H, 2 × $H_{I\beta}$), 1.46 – 1.39 (m, 2H, $H_{I\gamma1}$), 1.11 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3), 1.10 – 1.03 (m, 2H, $H_{I\gamma1}$), 0.85 (d, 3H, $J_{H\gamma,H\beta}$ = 6.8 Hz, $H_{I\gamma2}$), 0.83 (d, 3H, $J_{H\gamma,H\beta}$ = 6.8 Hz, $H_{I\gamma2}$) 0.81 (td, $J_{H\delta,H\gamma}$ = 7.4 Hz, $J_{H\delta,H\beta}$ = 2.7 Hz, 6H, 2 × $H_{I\delta}$). [ppm]

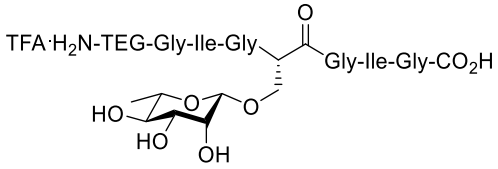
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (2C, 2 × C=O), 171.1 (C=O), 170.5 (C=O), 169.4 (C=O), 168.8 (2C, 2 × C=O), 168.3 (C=O), 99.6 (C-1), 71.9 (C-4), 70.5 (C-3), 70.2 (C-2), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 68.5 (C-5), 66.7 (2C, CH_2-Spacer), 66.3 (S_β), 56.8 (I_α), 56.7 (I_α), 52.4 (S_α), 42.0 (G_α), 41.9 (2C, 2 × G_α), 40.6 (G_α), 38.7 (CH_2-Spacer), 36.8 (I_β), 36.7 (I_β), 35.8 (CH_2-Spacer), 24.3 ($I_{\gamma1}$), 24.2 ($I_{\gamma1}$), 17.9 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 11.2 (2C, 2 × I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 171 Hz.

HRMS (ESI+): Calculated for $C_{38}H_{69}N_8O_{17}^+$ $[M+H]^+$: 909.4775; found: 909.4777.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 80% B, flow: 1ml/min**):** t_R = 11.07 min, λ = 230 nm.

Ser$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer (**S12**)



Glycopeptide was synthesized according to general procedure for 0.05 mmol scale peptide synthesis. α-Rhamnosyl serine building block **4** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Fmoc-TEG-CO_2H building block was coupled with a 4-fold excess and a coupling time of 1 h per coupling. Cleavage of the *O*-ester protecting groups was performed according to the general procedure for on resin-deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) yielded **S12** (20 mg, 20 µmol, 40%) after lyophilization.

^{1}H-NMR: (600 MHz, DMSO-d_6): δ = 8.35 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.24 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.21 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.09 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.06 (d, $J_{NH,H\alpha}$ = 7.8 Hz, 1H, NH_S), 7.84 (d, $J_{NH,H\alpha}$ = 8.7 Hz, 1H, NH_I), 7.80 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 7.74 (bs, 3H, NH_3^+), 4.45 – 4.40 (m, 2H, $H_{S\alpha}$, H-1), 4.26 – 4.18 (m, 2H, 2 × $H_{I\alpha}$), 3.86 – 3.65 (m, 11H, 8 × $H_{G\alpha}$, $H_{S\beta}$, H-2), 3.63 – 3.47 (m, 12H, 12 × CH_2-O-Spacer), 3.21 (dd, $J_{H3,H4}$ = 8.8 Hz, $J_{H3,H2}$ = 3.2 Hz, 1H, H-3), 3.13 – 3.07 (m, 2H, H-4, H-5), 3.01 – 2.98 (m, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.74 – 1.68 (m, 2H, 2 × $H_{I\beta}$), 1.45 – 1.40 (m, 2H, $H_{I\gamma1}$), 1.17 (d, $J_{CH3,H5}$ = 5.5 Hz, 3H, rha-CH_3), 1.10 – 1.01 (m, 2H, $H_{I\gamma1}$), 0.84 (t, $J_{H\gamma,H\beta}$ = 6.8 Hz, 6H, 2 × $H_{I\gamma2}$), 0.83 – 0.79 (m, 6H, 2 × $H_{I\delta}$). [ppm]

^{13}C-NMR (150 MHz, DMSO-d_6): δ = 171.3 (2C, 2 × C=O), 171.0 (C=O), 170.5 (C=O), 169.7 (C=O), 168.8 (C=O), 168.7 (C=O), 168.4 (C=O), 100.1 (C-1), 73.2 (C-3), 72.1 (C-4/C-5), 72.0

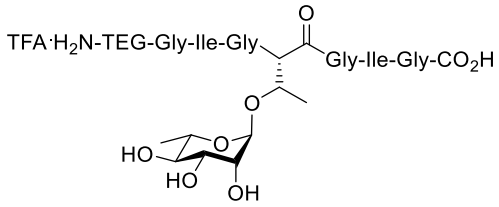
(C-4/C-5), 70.4 (C-2), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 68.1 (S_β), 66.7 (2C, CH_2-Spacer), 56.8 (I_α), 56.6 (I_α), 53.0 (S_α), 42.0 (G_α), 41.9 (G_α), 41.8 (G_α), 40.6 (G_α), 38.7 (CH_2-Spacer), 36.9 (I_β), 36.7 (I_β), 35.8 (CH_2-Spacer), 24.2 ($I_{\gamma1}$), 24.1 ($I_{\gamma1}$), 17.9 (CH_3-rha), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (2C, 2 × I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 159 Hz.

HRMS (ESI+): Calculated for $C_{38}H_{69}N_8O_{17}^+$ $[M+H]^+$: 909.4775; found: 909.4782

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 80% B, flow: 1ml/min**):** t_R = 12.16 min, λ = 230 nm.

Thr$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer (**S13)**



Glycopeptide was synthesized according to general procedure for 0.1 mmol scale peptide synthesis. α-Rhamnosyl threonine building block **2** was coupled following a double coupling protocol with 3-fold excess for each coupling step and coupling time of 1.5 h per coupling. Fmoc-TEG-CO_2H building block was coupled following a double coupling protocol with 4-fold excess for each coupling step and a coupling time of 1 h per coupling. Cleavage of the *O*-acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) yielded **S13** (52 mg, 50 µmol, 50%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.31 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.28 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.10 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.06 (t, $J_{NH,H\alpha}$ = 5.5 Hz, 1H, NH_G), 7.95 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_I), 7.85 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_I), 7.76 (s, 3H, NH_3^+), 7.74 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_T), 4.59 (d, $J_{H1,\,H2}$ = 1.6 Hz, 1H, H-1), 4.43 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 3.6 Hz, 1H, $H_{T\alpha}$), 4.21 (dd, $J_{NH,H\alpha}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.1 Hz, 2H, 2 × $H_{I\alpha}$), 4.10 – 4.04 (m, 1H, $H_{T\beta}$), 3.85 –

3.74 (m, 7H, 7 × $H_{Gα}$), 3.69 (dd, $J_{Hα,Hα}$ = 17.4 Hz, $J_{Hα,NH}$ = 5.8 Hz, 1H, $H_{Gα}$), 3.61 – 3.47 (m, 13H, 12 × CH_2-O-Spacer, H-2), 3.41 (dd, $J_{H3,H4}$ = 9.4 Hz, $J_{H3,H2}$ = 3.4 Hz, 1H, H-3), 3.33 (dq, $J_{H5,H4}$ = 9.4 Hz, $J_{H5,CH3}$ = 6.2 Hz, 1H, H-5), 3.14 (t, $J_{H4,H3}$ = $J_{H4,H5}$ =9.4 Hz, 1H, H-4), 2.98 (q, $J_{CH,CH}$ = 5.5 Hz, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.76 – 1.67 (m, 2H, 2 × $H_{Iβ}$), 1.46 – 1.40 (m, 2H, $H_{Iγ1}$), 1.11 – 1.03 (m, 5H, rha-CH_3, $H_{Iγ1}$), 1.02 (d, $J_{Hγ,Hβ}$ = 6.2 Hz, 3H, $H_{Tγ}$), 0.84 (t, $J_{Hγ,Hβ}$ = 7.3 Hz, 6H, 2 × $H_{Iγ2}$), 0.80 (td, $J_{Hδ,Hγ}$ = 7.4 Hz, $J_{Hδ,Hβ}$ = 3.4 Hz, 6H, 2 × $H_{Iδ}$). [ppm]

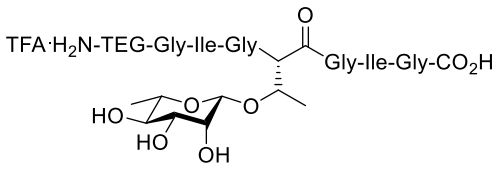
^{13}C-NMR (200 MHz, DMSO-d_6): δ [ppm] = 171.3 (2C, 2 × C=O), 171.1 (C=O), 170.5 (C=O), 169.5 (C=O), 169.0 (C=O), 168.7 (C=O), 168.4 (C=O), 96.0 (C-1), 71.9 (C-4), 70.7 (C-2), 70.5 (C-3), 69.7 (2C, CH_2-Spacer), 69.6 (2C, CH_2-Spacer, $T_β$), 69.5 (CH_2-Spacer), 68.6 (C-5), 66.7 (2C, CH_2-Spacer), 56.8 ($I_α$), 56.7 ($T_α$), 56.6 ($I_α$), 42.0 (2C, 2 × $G_α$), 41.9 ($G_α$), 40.6 ($G_α$), 38.7 (CH_2-Spacer), 36.8 (2C, 2 × $I_β$), 35.9 (CH_2-Spacer), 24.3 ($I_{γ1}$), 24.2 ($I_{γ1}$), 17.8 (rha-CH_3), 15.3 (2C, 2 × $I_{γ2}$), 14.5 ($T_γ$), 11.2 ($I_δ$), 11.1 ($I_δ$). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 170 Hz.

HRMS (ESI+): Calculated for $C_{39}H_{71}N_8O_{17}^+$ $[M+H]^+$: 923.4932; found: 923.4944.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 80% B, flow: 1ml/min**):** t_R = 12.07 min, λ = 230 nm.

Thr$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer (**S14)**



Glycopeptide was synthesized according to general procedure for 0.05 mmol scale peptide synthesis. β-Rhamnosyl threonine building block **5** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 1.5 h per coupling. Fmoc-TEG-CO_2H building block was coupled with a 4-fold excess for each coupling and a coupling time of 1 h per coupling. Cleavage of the *O*-ester protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the previously described procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) yielded **S14** (25 mg, 24 µmol, 48%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-d_6): δ = 8.32 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.29 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.16 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.06 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 7.91 (d, $J_{NH,H\alpha}$ = 8.9 Hz, 1H, NH_I), 7.88 (d, $J_{NH,H\alpha}$ = 8.7 Hz, 1H, NH_I), 7.78 (d, $J_{NH,H\alpha}$ = 8.8 Hz, 1H, NH_T), 7.75 (bs, 3H, NH_3^+), 4.44 (s, 1H H-1), 4.34 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 3.6 Hz, 1H, $H_{T\alpha}$), 4.23 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 7.0 Hz, 1H, NH_I), 4.20 (dd, $J_{H\alpha,NH}$ = 8.7 Hz, $J_{H\alpha,H\beta}$ = 7.2 Hz, 1H, NH_I), 4.10 (qd, $J_{H\beta,H\gamma}$ = 6.4 Hz, $J_{H\beta,H\alpha}$ = 3.5 Hz, 1H, $H_{T\beta}$), 3.87 – 3.66 (m, 8H, 8 × $H_{G\alpha}$), 3.64 (d, $J_{H2,H3}$ = 3.3 Hz, 1H, H-2), 3.62 – 3.47 (m, 12H, 12 × CH_2-O-Spacer), 3.18 (dd, $J_{H3,H4}$ = 9.0 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 3.08 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.1 Hz, 1H, H-4), 3.04 (dq, $J_{H5,H4}$ = 9.1 Hz, $J_{H5,CH3}$ = 5.9 Hz, 1H, H-5), 3.00 – 2.95 (m, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.75 – 1.68 (m, 2H, 2 × $H_{I\beta}$), 1.46 – 1.39 (m, 2H, $H_{I\gamma1}$), 1.15 (d, $J_{CH3,H5}$ = 5.9 Hz, 3H, rha-CH_3), 1.10 (d, $J_{H\beta,H\gamma}$ = 6.4 Hz, 3H, -$H_{T\gamma}$), 1.09 – 1.03 (m, 2H, $H_{I\gamma1}$), 0.85 (d, $J_{H\gamma,H\beta}$ = 6.9 Hz, 3H, $H_{I\gamma2}$), 0.84 (d, $J_{H\gamma,H\beta}$ = 6.8 Hz, 3H, $H_{I\gamma2}$), 0.82 – 0.79 (m, 6H, 2 × $H_{I\delta}$). [ppm]

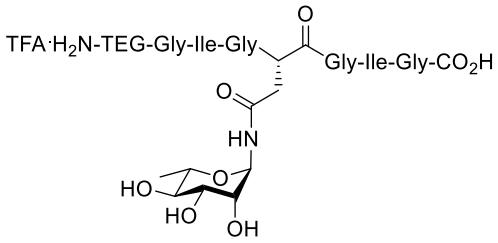
^{13}C-NMR (200 MHz, DMSO-d_6): δ = 171.3 (2C, 2 × C=O), 171.1 (C=O), 170.5 (C=O), 169.8 (C=O), 168.9 (2C, 2 × C=O), 168.4 (C=O), 100.7 (C-1), 75.2 (T_β), 73.1 (C-3), 72.0 (C-4/C-5), 71.9 (C-4/C-5), 70.6 (C-2), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 66.7 (2C, CH_2-Spacer), 56.8 (I_α), 56.7 (2C, I_α, T_α), 41.9 (2C, 2 × G_α), 41.8 (G_α), 40.6 (G_α), 38.7 (CH_2-Spacer), 36.9 (I_β), 36.7 (I_β), 35.9 (CH_2-Spacer), 24.2 ($I_{\gamma1}$), 24.1 ($I_{\gamma1}$), 18.8 (T_γ), 18.0 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 11.2 (I_δ), 11.1 (I_δ). [ppm]

^{1}H-^{13}C-HSQC (DMSO-d_6): $J_{H1,C1}$ = 156 Hz.

HRMS (ESI+): Calculated for $C_{39}H_{71}N_8O_{17}^+$ [M+H]$^+$: 923.4932; found: 923.4935.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 80% B, flow: 1ml/min**):** t_R = 12.52 min, λ = 230 nm.

Asn$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer (**S15**)



The synthesis of α-rhamnosyl asparagine glycopeptide **S15** was performed according to the general procedure for peptide synthesis on a 0.1 mmol scale. α-Rhamnosyl asparagine building block **9** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 2 h per coupling. Fmoc-TEG-CO_2H-Spacer was coupled following a double coupling protocol with 4-fold excess for each coupling and a coupling time of 1 h per coupling. Deprotection of the acetyl protecting groups was performed according to the general procedure for on-resin deprotection. Cleavage from resin according to the general procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) afforded **S15** (48 mg, 46 µmol, 46%) after lyophilization.

^{1}H-NMR (800 MHz, DMSO-D_6): δ = 8.50 (d, $J_{NH,\,H1}$ = 8.8 Hz, 1H, $NH_{N\gamma}$), 8.29 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.24 (t, $J_{NH,H\alpha}$ = 5.7 Hz, 1H, NH_G), 8.12 (d, $J_{NH,H\alpha}$ = 7.9 Hz, 1H, NH_N), 8.10 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.06 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 7.87 (d, $J_{NH,H\alpha}$ = 8.5 Hz, 1H, NH_I), 7.77 – 7.72 (m, 4H, NH_I, NH_3^+), 5.22 (dd, $J_{H1,NH}$ = 8.8 Hz, $J_{H1,H2}$ = 2.0 Hz, 1H, H-1), 4.55 (td, $J_{N\alpha,NH}$ = 7.8 Hz, $J_{H\alpha,H\beta}$ = 5.4 Hz, 1H, $H_{N\alpha}$), 4.22 – 4.18 (m, 2H, 2 × $H_{I\alpha}$), 3.80 – 3.68 (m, 8H, 8 × $H_{G\alpha}$), 3.63 – 3.48 (m, 14H, 6 × CH_2-O-Spacer, H-2, H-3), 3.39 – 3.35 (m, 1H, H-5), 3.20 (t, $J_{H4,H3}$ = $J_{H4,H5}$ = 9.0 Hz, 1H, H-4), 3.00 − 2.96 (m, 2H, CH_2-O-Spacer), 2.63 – 2.61 (m, 1H, $H_{N\beta}$), 2.54 – 2.52 (m, 1H, $H_{N\beta}$), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.76 – 1.69 (m, 2H, 2 × $H_{I\beta}$), 1.44 – 1.40 (m, 2H, $H_{I\gamma1}$), 1.09 (d, $J_{CH3,H5}$ = 6.2 Hz, 3H, rha-CH_3), 1.07 – 1.05 (m, 2H, $H_{I\gamma1}$), 0.85 – 0.79 (m, 12H, 2 × $I_{\gamma2}$, 2 × I_δ). [ppm]

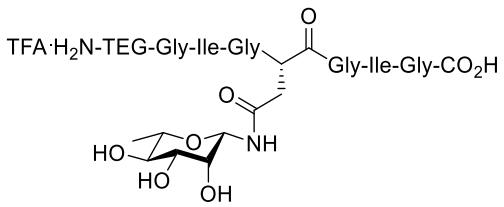
^{13}C-NMR (200 MHz, DMSO-D_6): δ = 171.4 (C=O), 171.3 (C=O), 171.1 (2C, 2 × C=O), 170.5 (C=O), 169.6 (C=O), 168.9 (C=O), 168.7 (C=O), 168.4 (C=O), 77.8 (C-1), 72.1 (C-4), 70.6 (C-2/C-3), 70.2 (C-2/C-3), 69.7 (2C, CH_2-O-Spacer), 69.6 (CH_2-O-Spacer), 69.5 (2C, C-5, CH_2-O-Spacer), 66.7 (2C, CH_2-O-Spacer), 56.9 (I_α), 56.6 (I_α), 49.7 (N_α), 42.1 (G_α), 41.9 (G_α), 41.8 (G_α), 40.6 (G_α), 38.7 (CH_2-O-Spacer), 37.3 (N_β), 36.7 (I_β), 36.6 (I_β), 35.8 (CH_2-O-Spacer), 24.3 ($I_{\gamma1}$), 24.2 ($I_{\gamma1}$), 18.0 (rha-CH_3), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (2C, 2 × I_δ). [ppm]

^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 162 Hz.

HRMS (ESI+): Calculated for $C_{39}H_{70}N_9O_{17}$ $[M+H]^+$: 936.4884; found: 936.4884.

RP-HPLC (Aeris, 0.1% TFA, 0 min: 5% B → 40 min: 80% B → 60 min: 100% B, flow: 1 ml/min): t_R = 11.41 min, λ = 230 nm.

Asn$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer (**S16**)



The synthesis of β-rhamnosyl asparagine glycopeptide **S16** was performed according to the general procedure for peptide synthesis on a 0.1 mmol scale. β-Rhamnosyl asparagine building block **7** was coupled following a double coupling protocol with 3-fold excess for each coupling step and a coupling time of 2 h per coupling. Fmoc-TEG-CO_2H-Spacer was coupled following a double coupling protocol with 5-fold excess for each coupling and a coupling time of 1 h per coupling. Deprotection of the acetyl protecting groups was performed according to the general procedure for deprotection. Cleavage from resin according to the general procedure furnished the crude glycopeptide. RP-HPLC purification (Gradient: A; λ = 205 nm) afforded **S16** (58 mg, 55 µmol, 55%) after lyophilization.

^{1}H-NMR (600 MHz, DMSO-D_6): δ = 8.26 – 8.17 (m, 3H, $NH_{N\gamma}$, 2 × NH_G), 8.14 – 8.08 (m, 3H, NH_N, 2 × NH_G), 7.86 (d, $J_{NH,I\alpha}$ = 8.6 Hz, 1H, NH_I), 7.78 (d, $J_{NH,I\alpha}$ = 8.9 Hz, 1H, NH_I),7.75 (bs, 3H,

NH_3^+), 4.98 (d, $J_{H1,NH}$ = 9.3 Hz, 1H, H-1), 4.56 (q, $J_{N\alpha,NH}$ = $J_{N\alpha,N\beta}$ = 7.1 Hz, 1H, $H_{N\alpha}$), 4.23 – 4.16 (m, 2H, 2 × $H_{I\alpha}$), 3.81 – 3.68 (m, 8H, 8 × $H_{G\alpha}$), 3.62 – 3.47 (m, 13H, H-2, 6 × CH_2-O-Spacer), 3.28 (dd, $J_{H3,H4}$ = 8.7 Hz, $J_{H3,H2}$ = 3.3 Hz, 1H, H-3), 3.14 – 3.06 (m, 2H, H-4, H-5), 3.02 − 2.94 (m, 2H, CH_2-O-Spacer), 2.66 (dd, $J_{H\beta,H\beta}$ = 15.9 Hz, $J_{H\beta,H\alpha}$ = 5.9 Hz, 1H, $H_{N\beta}$), 2.55 – 2.51 (m, 1H, $H_{N\beta}$), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.81-1.66 (m, 2H, 2 × $H_{I\beta}$), 1.48 – 1.38 (m, 2H, $H_{I\gamma1}$), 1.11 (d, $J_{CH3,H5}$ = 5.5 Hz, 3H, rha-CH_3), 1.11 – 1.03 (m, 2H, $H_{I\gamma1}$), 0.85 – 0.76 (m, 12H, 2 × $I_{\gamma2}$, 2 × I_δ). [ppm]

^{13}C-NMR (150 MHz, DMSO-D_6): δ = 171.3 (C=O), 171.2 (2C, 2 × C=O), 171.0 (C=O), 170.5 (C=O), 169.4 (C=O), 168.9 (C=O), 168.6 (C=O), 168.5 (C=O), 77.4 (C-1), 73.7 (C-3), 73.6 (C-4/C-5), 71.6 (C-4/C-5), 70.7 (C-2), 69.7 (2C, CH_2-O-Spacer), 69.6 (CH_2-O-Spacer), 69.5 (CH_2-O-Spacer), 66.7 (2C, CH_2-O-Spacer), 56.9 (I_α), 56.7 (I_α), 49.5 (N_α), 42.2 (G_α), 41.9 (G_α), 41.8 (G_α), 40.6 (G_α), 38.7 (CH_2-O-Spacer), 37.4 (N_β), 36.7 (I_β), 36.5 (I_β), 35.8 (CH_2-O-Spacer), 24.3 ($I_{\gamma1}$), 24.2 ($I_{\gamma1}$), 17.9 (rha-CH_3), 15.3 ($I_{\gamma2}$), 15.2 ($I_{\gamma2}$), 11.1 (I_δ), 11.0 (I_δ). [ppm]

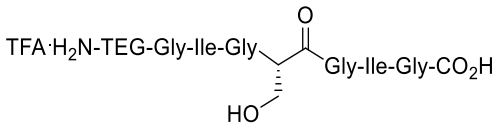
^{1}H-^{13}C-HSQC ($CDCl_3$): $J_{H1,C1}$ = 154 Hz

HRMS (ESI+): Calculated for $C_{39}H_{70}N_9O_{17}$ [M+H^+]: 936.4884; found 936.4873.

RP-HPLC (Aeris, 0.1% TFA, 0 min: 8% B → 10 min: 8% B → 60 min: 50% B → 70 min: 100% B; flow: 1 ml/ min): t_R = 11.79 min, λ = 230 nm.

Synthesis of non-glycosylated Peptides (Naked Peptides (NP))

Naked Ser-Peptide (**S17, Ser^{NP}**)



Naked Ser-Peptide **S17** was synthesized according to the general procedures for microwave assisted peptide synthesis. Cleavage from resin was conducted as described in the general procedure. Purification *via* RP-HPLC (Gradient: A; λ = 205 nm) furnished **S17** (32 mg, 37 µmol, 37%) after lyophilization.

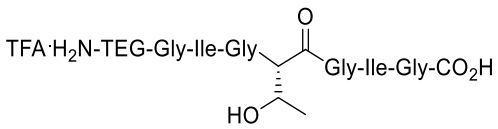
^{1}H-NMR (600 MHz, DMSO-d_6): δ = 8.33 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.28 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.18 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, -NH_G), 8.11 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 7.95 (d, $J_{NH,H\alpha}$ = 7.6 Hz, 1H, NH_S), 7.91 (d, $J_{NH,H\alpha}$ = 8.4 Hz, 1H, NH_I), 7.81 (s, 3H, NH_3^+), 7.77 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 4.25 (dt, $J_{H\alpha,NH}$ = 7.4 Hz, $J_{H\alpha,H\beta}$ = 5.5 Hz, 1H, $H_{S\alpha}$), 4.21 (dd, $J_{H\alpha,NH}$ = 8.9 Hz, $J_{H\alpha,H\beta}$ = 7.2 Hz, 1H, $H_{I\alpha}$), 4.17 (dd, $J_{H\alpha,NH}$ = 8.4 Hz, $J_{H\alpha,H\beta}$ = 7.2 Hz, 1H, $H_{I\alpha}$), 3.83 – 3.67 (m, 8H, 8 × $H_{G\alpha}$), 3.65 – 3.46 (m, 14H, 2 × $H_{S\beta}$, 12 × CH_2-O-Spacer), 3.01 – 2.93 (m, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.76 – 1.66 (m, 2H, 2 × $H_{I\beta}$), 1.47 – 1.38 (m, 2H, $H_{I\gamma1}$), 1.11 – 1.01 (m, 2H, $H_{I\gamma1}$), 0.88 – 0.75 (m, 12H, 2 × $H_{I\gamma2}$, 2 × $H_{I\delta}$). [ppm]

^{13}C-NMR (150 MHz, DMSO-d_6): δ = 171.3 (C=O), 171.2 (C=O), 171.0 (C=O), 170.5 (C=O), 170.4 (C=O), 169.0 (C=O), 168.8 (C=O), 168.5 (C=O), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 66.7 (2C, CH_2-Spacer), 61.8 (S_β), 57.1 (I_α), 56.6 (I_α), 55.3 (S_α), 42.1 (G_α), 41.9 (G_α), 41.8 (G_α), 40.6 (G_α), 38.6 (CH_2-Spacer), 36.8 (I_β), 36.6 (I_β), 35.8 (CH_2-Spacer), 24.3 ($I_{\gamma1}$), 24.1 ($I_{\gamma1}$), 15.3 ($I_{\gamma2}$), 15.2 ($I_{\gamma2}$), 11.1 (2C, 2 × I_δ). [ppm]

HRMS (ESI+): Calculated for $C_{32}H_{59}N_8O_{13}^+$ $[M+H]^+$: 763.4196; found: 763.4197.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min): t_R = 12.09 min, λ = 230 nm.

Naked Thr-Peptide (**S18, Thr^{NP}**)



Naked Thr-Peptide **S18** was synthesized according to the general procedures for microwave assisted peptide synthesis. Cleavage from resin was conducted as described in the general procedure. Purification *via* RP-HPLC (Gradient: A; λ = 205 nm) yielded **S18** (42 mg, 47 µmol, 47%) after lyophilization.

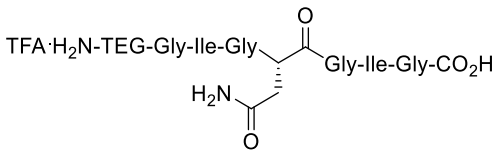
^{1}H-NMR (800 MHz, $CDCl_3$): δ = 8.32 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.25 (bs, 1H, NH_G), 8.15 – 8.10 (m, 2H, 2 × NH_G), 7.99 (d, $J_{NH,H\alpha}$ = 6.4 Hz, 1H, NH_T), 7.81 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 7.78 (d, $J_{NH,H\alpha}$ = 8.2 Hz, 1H, NH_I), 4.21 (dd, $J_{H\alpha,NH}$ = 9.0 Hz, $J_{H\alpha,H\beta}$ = 7.3 Hz, 1H, $H_{I\alpha}$), 4.19 – 4.16 (m, 2H, $H_{I\alpha}$, $H_{T\alpha}$), 4.02 – 3.98 (m, 1H, $H_{T\beta}$), 3.82 – 3.67 (m, 8H, 8 × $H_{G\alpha}$), 3.62 – 3.46 (m, 12H, 12 × CH_2-O-Spacer), 2.98 (t, $J_{CH,CH}$ = 5.2 Hz, 2H, CH_2-O-Spacer), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.75 – 1.69 (m, 2H, 2 × $H_{I\beta}$), 1.47 – 1.39 (m, 2H, $H_{I\gamma1}$), 1.11 – 1.01 (m, 5H, $H_{T\gamma}$, $H_{I\gamma1}$), 0.85 – 0.82 (m, 6H, 2 × $H_{I\gamma2}$), 0.81 – 0.78 (m, 6H, 2 × $H_{I\delta}$). [ppm]

^{13}C-NMR (200 MHz, $CDCl_3$): δ= 171.3 (C=O), 171.2 (C=O), 171.1 (C=O), 170.5 (2C, 2 × C=O), 169.0 (2C, 2 × C=O), 168.6 (C=O), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 66.7 (3C, CH_2-Spacer, T_β), 58.5 (T_α), 57.0 (I_α), 56.7 (I_α), 42.1 (2C, 2 × G_α), 41.9 (G_α), 40.8 (G_α), 38.7 (CH_2-Spacer), 36.7 (I_β), 36.6 (I_β), 35.8 (CH_2-Spacer), 24.3 ($I_{\gamma1}$), 24.1 ($I_{\gamma1}$), 19.5 (T_γ), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (2C, 2 × I_δ). [ppm]

HRMS (ESI+): Calculated for $C_{33}H_{61}O_{13}N_8^+$ $[M+H]^+$: 777.4353; found: 777.4341.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min): t_R = 12.09 min, λ = 230 nm.

Naked Asn-Peptide (**S19, Asn^{NP})**



Naked Asn-Peptide **S19** was synthesized according to the general procedures for microwave assisted peptide synthesis. Cleavage from resin was conducted as described in the general procedure for cleavage from resin. Subsequent purification *via* RP-HPLC (Gradient: A; λ = 205) yielded **S19** (35 mg, 39 µmol, 39%) after lyophilization.

^{1}H-NMR: (600 MHz, DMSO-d_6): δ = 8.28 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.25 (t, $J_{NH,H\alpha}$ = 5.9 Hz, 1H, NH_G), 8.13 (t, $J_{NH,H\alpha}$ = 5.8 Hz, 1H, NH_G), 8.11 – 8.07 (m, 2H, NH_G, NH_N), 7.93 (d, $J_{NH,H\alpha}$ = 8.4 Hz, 1H, NH_I), 7.76 (d, $J_{NH,H\alpha}$ = 9.0 Hz, 1H, NH_I), 7.39 (s, 1H, NH_N), 6.91 (s, 1H, NH_N), 4.52 (td, $J_{H\alpha,H\beta}$ = 7.5 Hz, $J_{H\alpha,NH}$ = 5.7 Hz, 1H, $H_{N\alpha}$), 4.21 – 4.15 (m, 2H, 2 × $H_{I\alpha}$), 3.81 – 3.66 (m, 8H, 8 × $H_{G\alpha}$), 3.62 – 3.47 (m, 12H, CH_2-O-Spacer), 2.98 (t, $J_{CH,CH}$ = 5.2 Hz, 2H, CH_2-O-Spacer), 2.56 – 2.52 (m, 1H, $H_{N\beta}$), 2.45 (dd, $J_{H\beta,H\beta}$ = 15.6 Hz, $J_{H\beta,H\alpha}$ = 7.2 Hz, 1H, $H_{N\beta}$), 2.38 (t, $J_{CH,CH}$ = 6.5 Hz, 2H, CH_2-O-Spacer), 1.80 – 1.67 (m, 2H, 2 × $H_{I\beta}$), 1.49-1.38 (m, 2H, $H_{I\gamma1}$), 1.13-1.01 (m, 2H, $H_{I\gamma1}$), 0.90 – 0.76 (m, 12H, 2 × $H_{I\gamma2}$, 2 × $H_{I\delta}$). [ppm]

^{13}C-NMR (150 MHz, DMSO-d_6): δ [ppm] = 171.6 (C=O), 171.4 (C=O), 171.2 (C=O), 171.1 (C=O), 170.5 (C=O), 168.9 (C=O), 168.8 (C=O), 168.5 (C=O), 69.7 (2C, CH_2-Spacer), 69.6 (CH_2-Spacer), 69.5 (CH_2-Spacer), 66.7 (2C, CH_2-Spacer), 57.0 (I_α), 56.8 (I_α), 49.7 (N_α), 42.2 (G_α), 42.0 (G_α), 41.9 (G_α), 40.7 (G_α), 38.7 (CH_2-Spacer), 37.1 (N_β), 36.6 (2C, I_β), 35.8 (CH_2-Spacer), 24.3 ($I_{\gamma1}$), 24.2 ($I_{\gamma1}$), 15.3 (2C, 2 × $I_{\gamma2}$), 11.1 (I_δ), 11.0 (I_δ). [ppm]

Note: Due to signal overlap C-terminal carbonyl could not be assigned in the ^{13}C-NMR.

HRMS (ESI+): Calculated for $C_{33}H_{60}N_9O_{13}$ $[M+H]^+$: 790.4305; found: 790.4308.

RP-HPLC (Aeris, 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min): t_R = 12.09 min, λ = 230 nm.

General Procedure for BSA Conjugation

Synthesis of squarate monoamides was conducted according to a slightly modified procedure.[18]

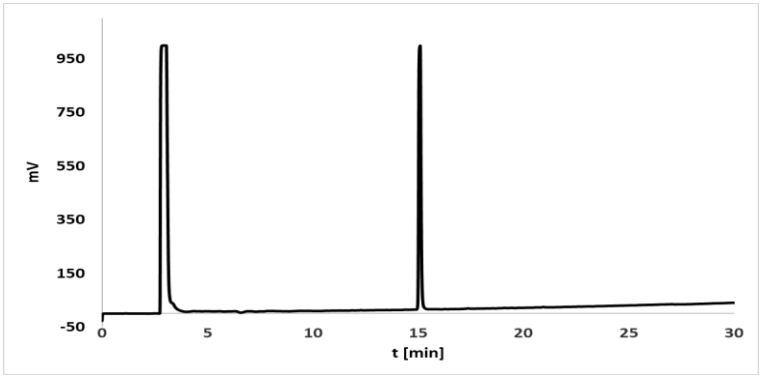
To a magnetically stirred solution of peptide (1.0 eq.) in a mixture of H_2O/EtOH (v/v = 1:1), 3,4-diethoxy-3-cyclobutene-1,2-dione (1.3 eq.) was added. The pH-value was carefully adjusted to pH = 8 by addition of a saturated Na_2CO_3 solution. The reaction was stirred until complete conversion of the starting material was observed by RP-HPLC monitoring. Subsequently the reaction was neutralized by careful addition of 1 M AcOH and lyophilized. The crude product was subjected to RP-HPLC (Gradient: A) and lyophilized, to furnish the desired squarate monoamide as colourless lyophilizate.

Table 4: Experimental data of glycopeptide mono amides for BSA conjugation

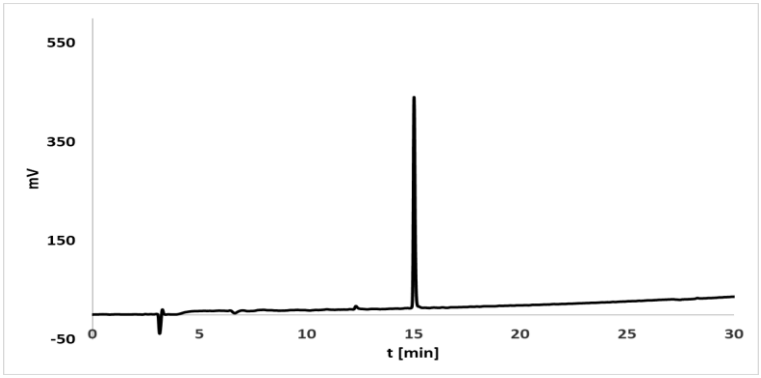
Peptide	Monoamide	ESI-MS [m/z]	RP-HPLC*
$Ser^{\alpha\text{-}Rha}$-GP (**S11**) (34 mg, 37 µmol)	**S20** 16 mg (16 µmol, 43%)	Calc.: $[M+NH_4^+]$: 1050.5201; found: 1050.5227.	t_R = 15.07 min λ = 230 nm
Ser^{β}-Rha-GP (**S12**) (15 mg, 17 µmol)	**S21** 8 mg (8 µmol, 47%)	Calc.: $[M+NH_4^+]$: 1050.5201; found: 1050.5217	t_R = 15.13 min λ = 230 nm
$Thr^{\alpha\text{-}Rha}$-GP (**S13**) (37 mg, 40 µmol)	**S22** 21 mg (20 µmol, 50%)	Calc.: $[M+NH_4^+]$: 1064.5358; found: 1064.5374.	t_R = 15.11 min λ = 230 nm
$Thr^{\beta\text{-}Rha}$-GP (**S14**) (15 mg, 16 µmol)	**S23** 6 mg (6 µmol, 38%)	Calc.: $[M+NH_4^+]$: 1064.5358; found: 1064.5377.	t_R = 15.44 min λ = 230 nm
$Asn^{\alpha\text{-}Rha}$-GP (**S15**) (24 mg, 23 µmol)	**S24** 11 mg (10 µmol, 44%)	Calc.: $[M+Na^+]$: 1082.4864; found: 1082.4864	t_R = 14.63 min λ = 230 nm
Asn^{β}-Rha-GP (**S16**) (40 mg, 38 µmol)	**S25** 22 mg (21 µmol, 55%)	Calc.: $[M+Na^+]$: 1082.4864; found: 1082.4874	t_R = 14.84 min λ = 230 nm
Ser^{NP} (**S17**) (30 mg, 39 µmol)	**S26** 18 mg (20 µmol, 51%)	Calc.: $[M+NH_4^+]$: 904.4622; found: 904.4631.	t_R = 15.61 min λ = 230 nm
Thr^{NP} (**S18**) (35 mg, 45 µmol)	**S27** 15 mg (17 µmol, 38%)	Calc.: $[M+NH_4^+]$: 918.4779; found: 918.4789.	t_R = 15.90 min λ = 230 nm
Asn^{NP} (**S19**) (61 mg, 70 µmol)	**S28** 26 mg (28 µmol, 40%)	Calc.: [M+Na+]: 936. 4285; found: 936.4275	t_R = 15.49 min λ = 230 nm

* Gradient: 0.1% TFA; 0 min 5% B → 40 min 80% B, flow: 1ml/min; Column: Phenomenex Aeris Peptide column (C18, 5 µm, 250 mm × 4.6 mm).

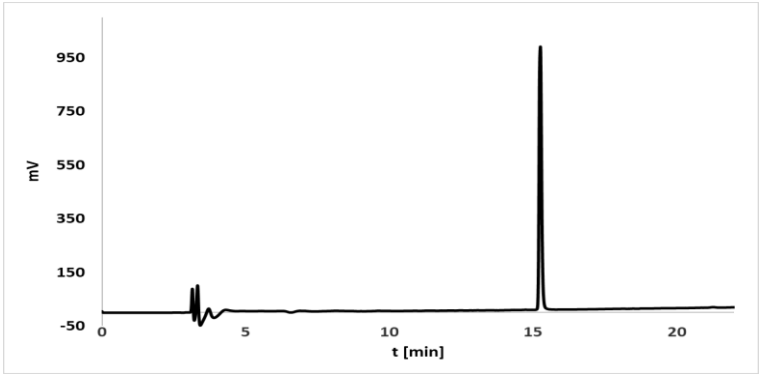
HPLC-Traces of purified squarate monoamides:



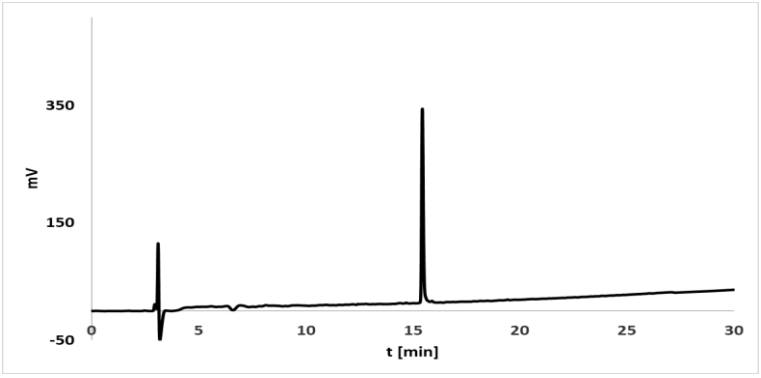
HPLC Trace of Ser$^{\alpha\text{-Rha}}$ peptide Squarate monoamide (**S20**); t_R = 2.81 min = DMSO



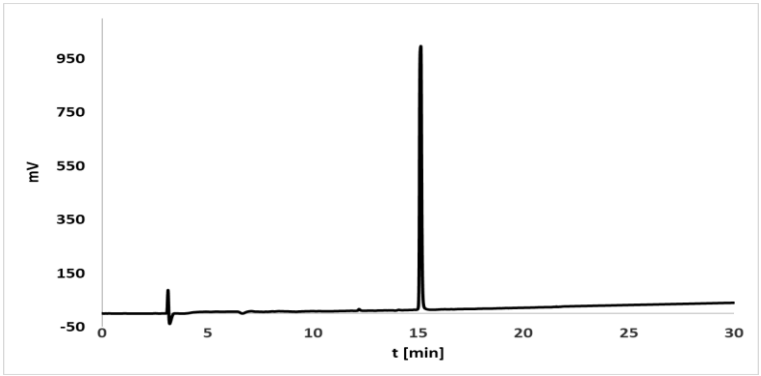
HPLC-Trace of Ser$^{\beta\text{-Rha}}$ Peptide squarate monoamide (**S21**)



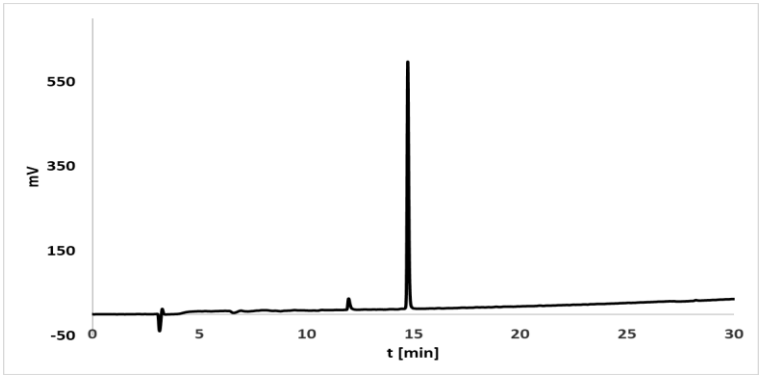
HPLC-Trace of Thr$^{\alpha\text{-Rha}}$ Peptide squarate monoamide (**S22**)



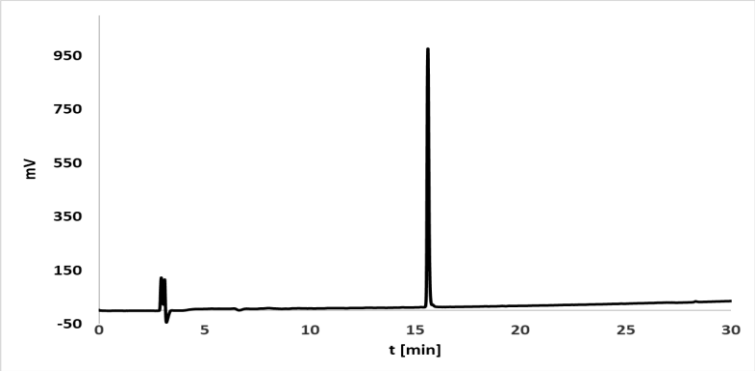
HPLC-Trace of Thr$^{\beta\text{-Rha}}$ Peptide squarate monoamide (**S23**)



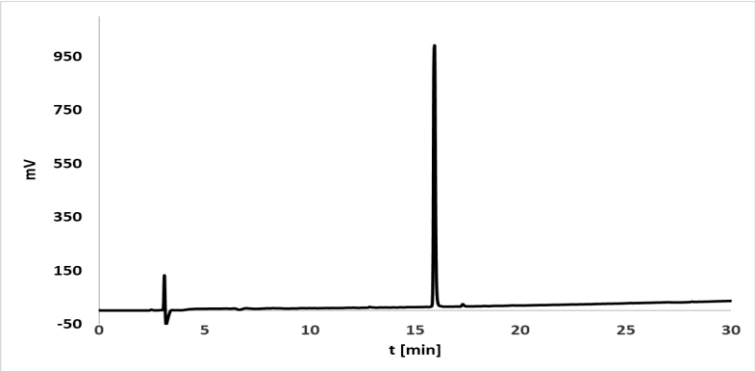
HPLC-Trace of Asn$^{\alpha\text{-rha}}$ peptide squarate monoamide (**S24**)



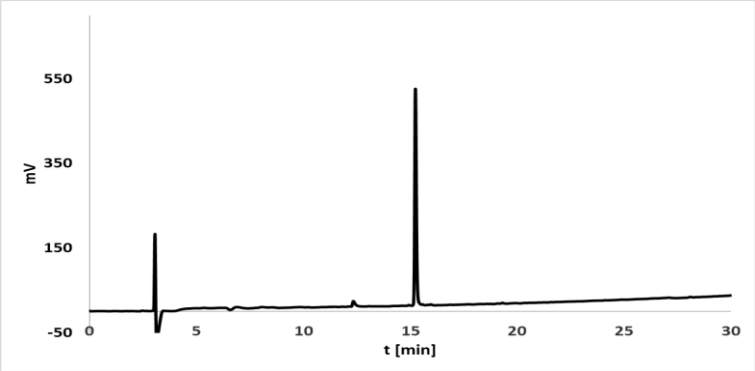
HPLC-Trace of Asn$^{\beta\text{-Rha}}$ peptide squarate monoamide (**S25**)



HPLC-Trace of Ser^{NP} squarate monoamide (**S26**)



HPLC-Trace of Thr^{NP} squarate monoamide (**S27**)



HPLC-Trace of Asn^{NP} squarate monoamide (**S28)**

Conjugation protocol:

BSA conjugation was conducted following a previously reported procedure.[18]

For the synthesis of BSA conjugates, the corresponding peptide squaric acid monoamide (2.0 µmol, 25 eq.) was dissolved in 1 ml of disodium phosphate buffer (65 mg Na_2HPO_4 per 1 ml of H_2O, pH = 9.5) and added to a solution of BSA (5 mg, 0.08 µmol, 1.0 eq.) in 1 ml of the same buffer. The mixture was agitated 3 d at 26 °C, before being filtered over an Amicon Ultra-15 centrifugal filter using a 10 kDa membrane and washed with water until the filtrate was neutral. The residue was lyophilized yielding the corresponding BSA conjugate.

Table 5: Experimental data for BSA conjugation

Monoamide	Conjugate	Yield	Loading [peptide/BSA]
S20 ($Ser^{\alpha\text{-}Rha}$)	**BSA-$Ser^{\alpha\text{-}Rha}$**	6 mg	14
S21 ($Ser^{\beta\text{-}Rha}$)	**BSA-$Ser^{\beta\text{-}Rha}$**	6 mg	18
S22 ($Thr^{\alpha\text{-}Rha}$)	**BSA-$Thr^{\alpha\text{-}Rha}$**	6 mg	17
S23 ($Thr^{\beta\text{-}Rha}$)	**BSA-$Thr^{\beta\text{-}Rha}$**	6 mg	17
S24 ($Asn^{\alpha\text{-}Rha}$)	**BSA-$Asn^{\alpha\text{-}Rha}$**	6 mg	22
S25 ($Asn^{\beta\text{-}Rha}$)	**BSA-$Asn^{\beta\text{-}Rha}$**	6 mg	20
S26 (Ser^{NP})	**BSA-Ser^{NP}**	6 mg	14
S27 (Thr^{NP})	**BSA-Thr^{NP}**	6 mg	17
S28 (Asn^{NP})	**BSA-Asn^{NP}**	6 mg	18

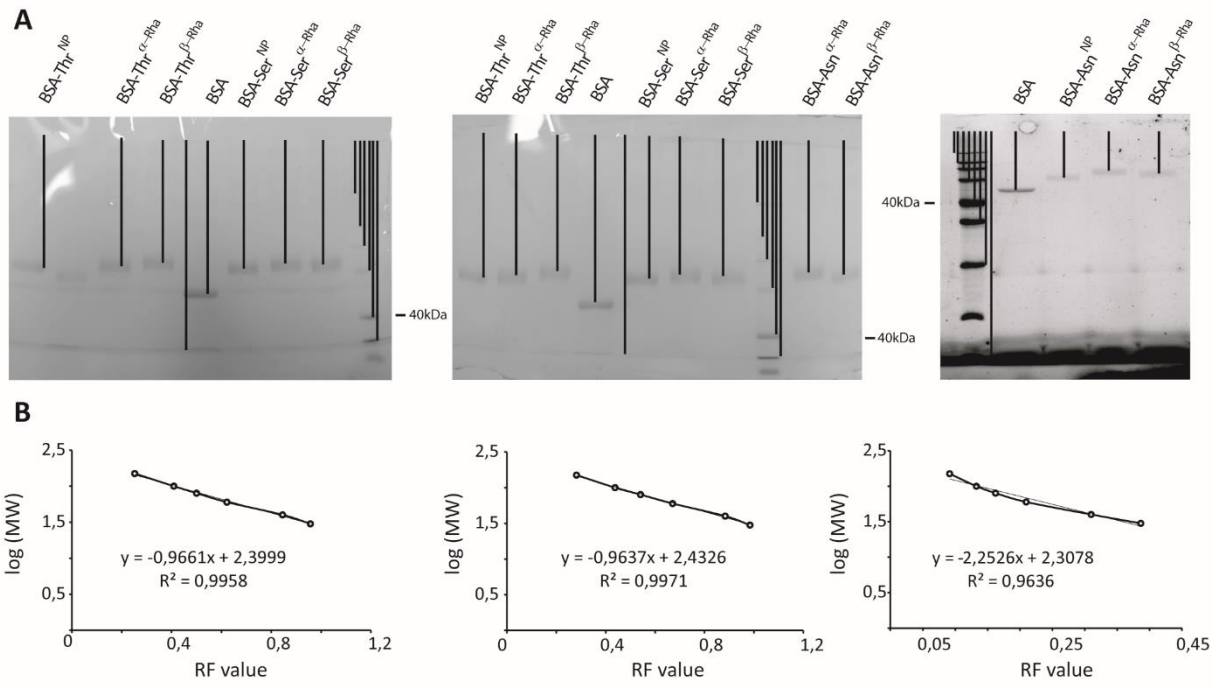


Figure 5: A) SDS-PAGE of aa^{Rha} conjugates. 0.5 µg of BSA, BSA coupled to naked peptide (BSA-aa^{NP}) and BSA conjugates of the corresponding rhamnosylated glycopeptides (BSA-$aa^{\alpha\text{-}/\beta\text{-}Rha}$) were subjected to SDS-PAGE and subsequent staining with InstantBlue™ (Expedeon Ltd.). The R_f values were calculated based on the ROTI®Mark 10-150 (Roth) molecular weight standard. The reference size of 40kDa is depicted. **B**) Graphics of linear equation for determining the R_F values. The linear equation was calculated from the protein ladder from the corresponding SDS-PAGE. The mean value of rhamnosylated peptide per BSA molecule was calculated using the linear equation.

Experimental Procedures for sensitivity and specificity evaluation of Antibodies

Specificity of *anti*-Asn^{Rha}, *anti*-Ser^{Rha} and *anti*-Thr^{Rha}

The specificity of the antibodies was determined by a Western Blot. For this purpose, 0.5 µg of each BSA, BSA-aa^{NP}, EF-P-aa^{Rha} and BSA-aa$^{\alpha-/\beta-Rha}$ were subjected to SDS-PAGE and Western Blot analysis with the corresponding antibody (0.2 µg/ml).

Antigen detection limits were determined by subjecting decreasing amounts of BSA-aa$^{\alpha-/\beta-Rha}$ (500 ng to 0.5 ng) to SDS-PAGE and Western Blot analysis as described above and detection with 0.2 µg/ml of the corresponding antibody.

To investigate cross-reactivity of the antibodies against free L-arginine, L-asparagine, L-serine, L-threonine or L-rhamnose, varying concentrations of BSA-aa$^{\alpha-/\beta-Rha}$ (0.2 µM to 150 µM) and putative competitors (5 mM, 15 mM) were preincubated with the corresponding antibody prior to BSA-aa$^{\alpha-/\beta-Rha}$ (0.5 µg) detection.

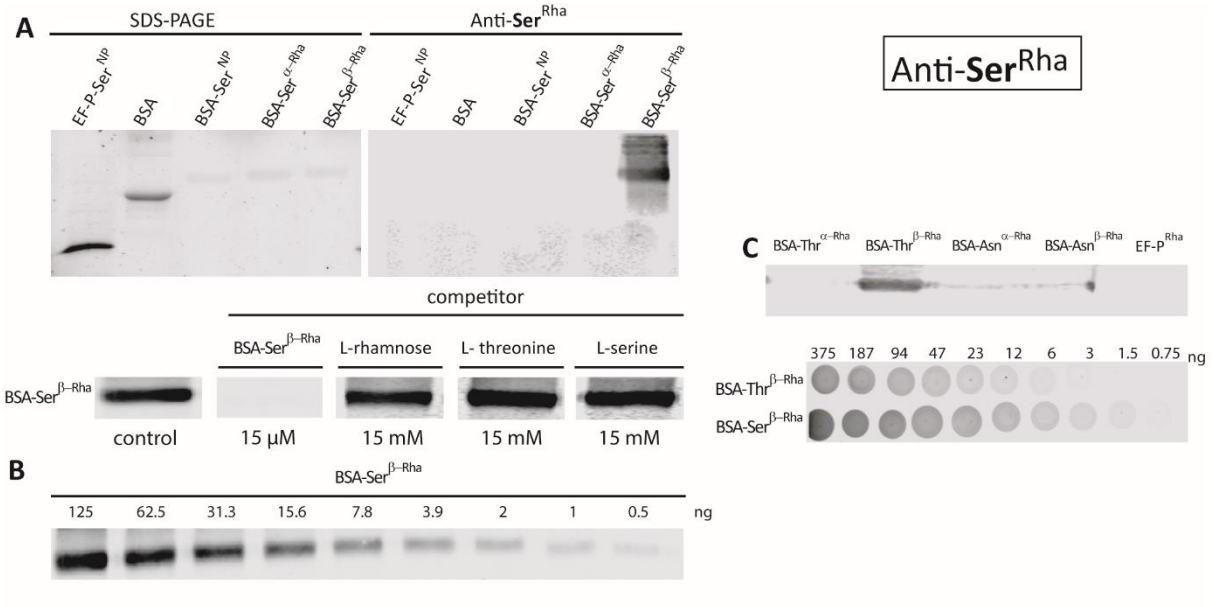


Figure 6: A) *Anti*-Ser^{Rha} specificity analysis: 0.5 µg of the respective sample were subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Ser^{Rha}. Upper part: BSA, EF-P-Ser^{Rha} and BSA coupled to the naked peptide (BSA-Ser^{NP}). BSA-Ser^{NP} served as negative controls. α- and β- rhamnosylated peptides coupled to BSA (BSA-Ser$^{\alpha-/\beta-Rha}$) served as positive controls. Lower part: Cross-reactivity analysis of *anti*-Ser^{Rha} against L-rhamnose, L-threonine and L-serine. *Anti*-Ser^{Rha} was preincubated prior to immunodetection with α- /β-Ser^{Rha} (15 µM), L-rhamnose, L-threonine and L-serine (15 mM). **B**) *Anti*-Ser^{Rha} sensitivity analysis of varying concentrations BSA-Ser$^{\alpha-/\beta-Rha}$. Antibody concentrations were kept constant at 0.2 mg/ml. **C**) Cross-reactivity analysis of *anti*-Ser^{Rha} against BSA-Thr$^{\alpha-/\beta-Rha}$, BSA-Asn$^{\alpha-/\beta-Rha}$ and EF-P^{Rha}. 0.5 µg of the respective sample were subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Ser^{Rha}.

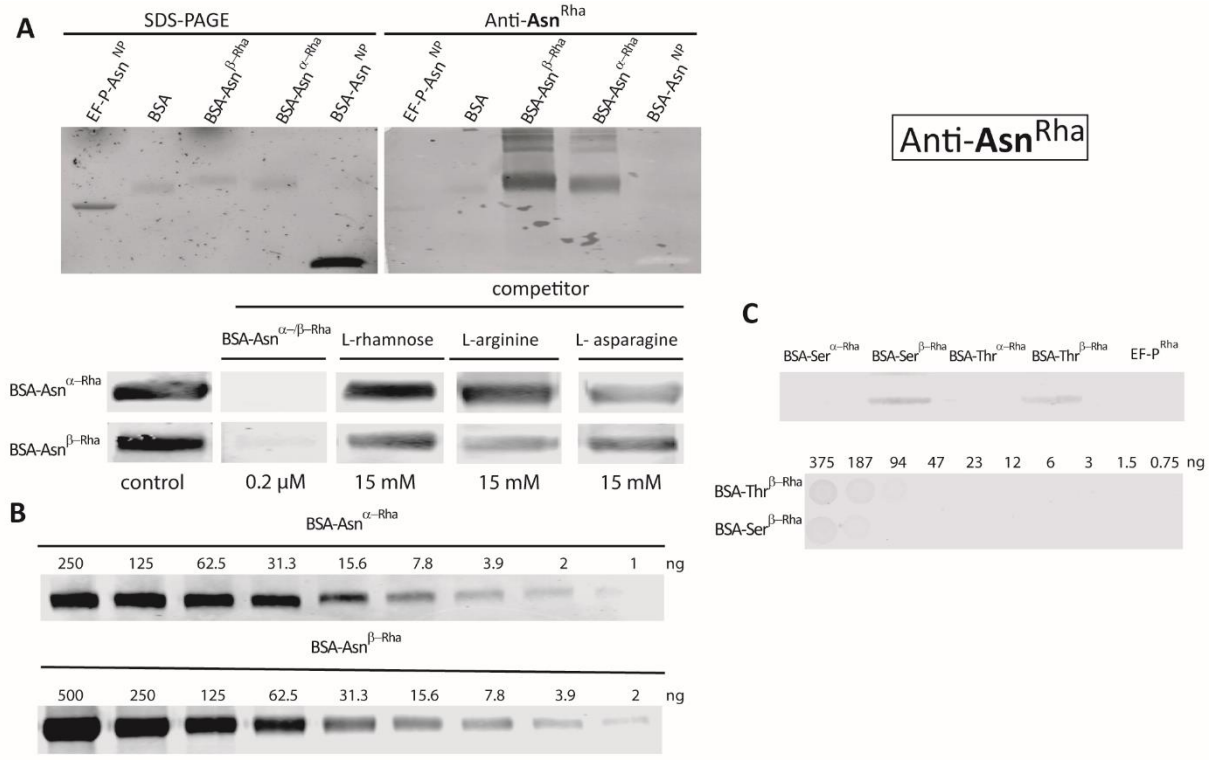


Figure 7: A) *Anti*-Asn^{Rha} specificity analysis: 0.5 µg of the respective sample were subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Asn^{Rha}. Upper part: BSA, EF-P-Asn^{Rha} and BSA coupled to the naked peptide (BSA-Asn^{NP}). BSA-Asn^{NP} served as negative controls. α- and β- rhamnosylated peptides coupled to BSA (BSA-Asn$^{α-/β-Rha}$) served as positive controls. Lower part: Cross-reactivity analysis of *anti*-Asn^{Rha} against L-rhamnose, L-asparagine and L-arginine. *Anti*-Asn^{Rha} was preincubated prior to immunodetection with BSA-Asn$^{α-/β-Rha}$ (0.2 µM), L-rhamnose, L-asparagine and L-arginine (15 mM). **B**) *Anti*-Asn^{Rha} sensitivity analysis of varying concentrations of BSA-Asn$^{α-/β-Rha}$. Antibody concentrations were kept constant at 0.2 mg/ml. **C**) Cross-reactivity analysis of *anti*-Asn^{Rha} against BSA-Ser$^{α-/β-Rha}$, BSA-Thr$^{α-/β-Rha}$ and EF-P^{Rha}. 0.5 µg of the respective sample were subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Asn^{Rha}.

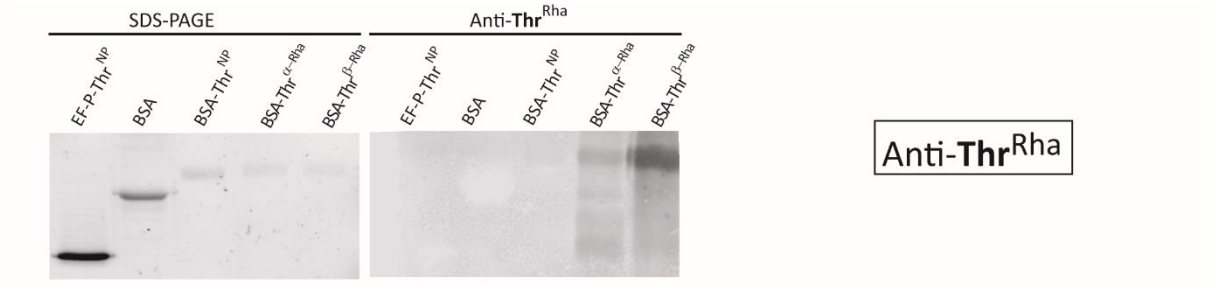


Figure 8: Anti-Thr^{Rha} specificity analysis: 0.5 µg of the respective sample were subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of anti-Thr^{Rha}. Upper part: BSA, EF-P-Thr^{Rha} and BSA coupled to the naked peptide BSA-Thr^{NP} served as negative controls. α- and β- rhamnosylated peptides coupled to BSA (BSA-Thr$^{α-/β-Rha}$) served as positive controls.

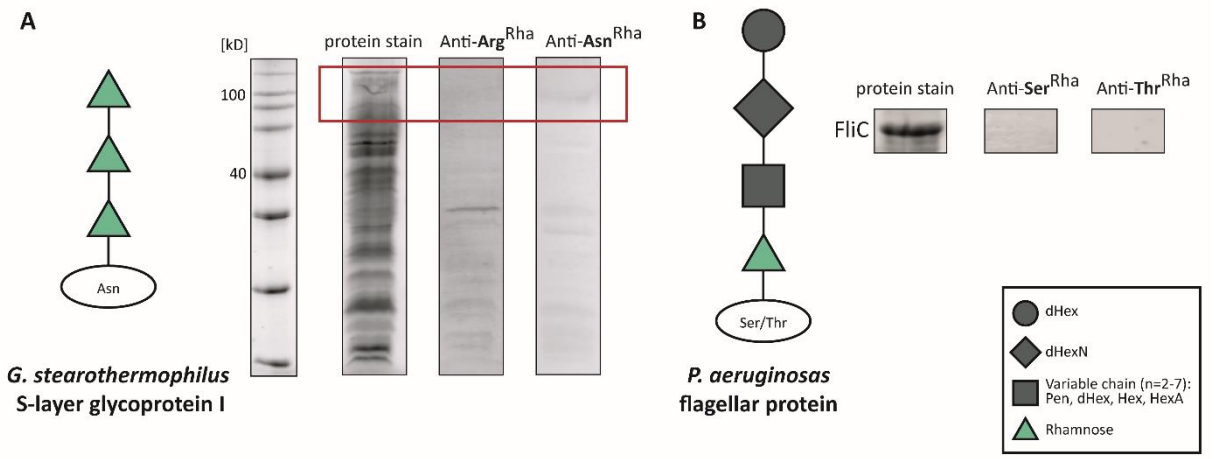


Figure 9: A) Immunodetection of *Geobacillus stearothermophilus* S-layer glycoprotein I. Left: Illustration of the glycanstructure of S-layer glycoprotein I. The S-layer glycoprotein I is poly-rhamnosylated (trisaccharide repeat of rhamnose) at an asparagine residue.[19] Right: The membrane fraction of *G. stearothermophilus,* which contains the poly-rhamnosylated S-layer glycoprotein, was subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Arg^{Rha} and *anti*-Asn^{Rha}. The S-layer glycoprotein revealed four bands of apparent molecular masses 93, 120, 147 and 170 kDa (area indicated by the red box).[19] **B**) Immunodetection of *Pseudomonas aeruginosa* flagellar protein. Left: Illustration of flagellar glycan structure. The protein is glycosylated at a serine and threonine residue (S260 and T189). A glycan comprising up to 11 monosaccharides units is *O* linked through a rhamnose residue to the protein.[20] Right: The flagellar protein was subjected to SDS-PAGE and subsequent Western Blot analysis with 0.2 mg/ml of *anti*-Ser^{Rha} and *anti*-Thr^{Rha}.

Detection of rhamnosylated proteins in cell lysates

Detection of rhamnosylated proteins in different organisms was carried out using total cell, cytosolic and membrane fractions. These were subjected to SDS-PAGE (total cell fraction: 10 µl, cytosolic fraction 10 µl, membrane fraction: 5 µl (OD600 of 25) and Western Blot analysis as described above.

Bacterial strains and growth conditions

Cells were grown in liquid media according to the growth conditions listed in table 6. When required, media were solidified by using 1.5% (w/v) agar. The optical density at 600 nm (OD600) of cultures was monitored and cells were harvested at OD600.

Table 6: Bacterial strains and growth conditions

Taxonomy	Strain	Culture condition/Feature	Source
Actinobacteria	*Corynebacterium glutamicum*	BHI medium (DSMZ 215)[21], 30 °C	DSM20300[21]
Actinobacteria	*Mycobacterium phlei*	Lysogeny broth (LB)[22], 37 °C	DSM43239[21]
Actinobacteria	*Micrococcus luteus*	LB, 30 °C	DSM20030[21]
Actinobacteria	*Streptomyces coelicolor*	LB + glycin (0.5 %), 30 °C	DSM40233[21]
Actinobacteria	*Streptomyces griseus*	LB + glycin (0.5 %), 30 °C	DSM40395[21]
Actinobacteria	*Streptomyces venezuelae*	LB + glycin (0.5 %), 30 °C	DSM40230[21]
Alphaproteobacteria	*Caulobacter crescentus*	Caulobacter medium (DSMZ 595)[21], 30 °C	DSM25117[21]
Firmicutes	*Bacillus subtilis*	LB, 30 °C	DSM10[21]
Firmicutes	*Geobacillus stearothermophilus*	LB, 55 °C	DSM22[21]
Firmicutes	*Staphylococcus carnosus*	HD + glycin (0.5 %), 37 °C	DSM20501[21]
Firmicutes	*Streptococcus salivarius*	HD + glycin (0.5 %), 37 °C	DSM20618[21]
Gammaproteobacteria	*Escherichia coli* BL21	LB, 37 °C	[23]
Gammaproteobacteria	*Escherichia coli* BW25113	LB, 37 °C	[24]
Gammaproteobacteria	*Escherichia coli* DH5αλpir	LB, 37 °C / recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 ΔlacZYA-argF U169 ϕ80dlacZΔM15 λpir	[25]
Gammaproteobacteria	*Escherichia coli* LMG194	LB, 37 °C / F- ΔlacX74 galE galK thi rpsL ΔphoA (PvuII) Δara714 leu Tn10	[26]
Gammaproteobacteria	*Escherichia coli* MG1655	LB, 37 °C	DSM18039[21]
Gammaproteobacteria	*Escherichia coli* Nissle 1917	LB, 37 °C	DSM6601[21]
Gammaproteobacteria	*Salmonella enterica* Typhimurium	LB, 37 °C	[27]

Taxonomy	Strain	Culture condition/Feature	Source
Gammaproteobacteria	*Shewanella oneidensis*	LB, 30 °C	ATCC® 700550™[28]
Gammaproteobacteria	*Pseudomonas aeruginosa*	LB, 37 °C	DSM22644[21]
Gammaproteobacteria	*Pseudomonas putida*	LB, 37 °C	DSM100120[21]
Deltaproteobacteria	*Myxococcus xanthus*	CTTYE medium[29], 32 °C	DSM16526[21]
Euryarchaeota	*Haloferax volcanii*	YPC[30], 45 °C	[31]
Sulfolobaceae	*Sulfolobus acidocaldarius*	Brock media[32], 76 °C	DSM639[21]
Cyanobacteria	*Synechococcus elongatus* PCC_6803	Cyanobacteria medium BG11 (DSMZ 1592)[21], 30 °C	[33]

Isolation of cytosolic and membrane fraction

Cell pellets were resuspended to a final O.D. of 25. Cell disruption was achieved for gram negative bacteria by Constant Systems Ltd. continuous-flow cabinet at 1.35 kb. Gram positive bacteria were treated with lysozyme (10 mg/ml) and further lysed by sonication. Cell debris were removed by centrifugation, cytosolic and membrane fraction were separated by ultracentrifugation.

Flagella isolation

Bacterial strains were grown overnight on agar plates (media and growth temperature according to table x). Cells from two plates were resuspended in 2 ml of phosphate-buffered saline (PBS, pH 7.4). The homogenate was passaged 20 times through a needle (Sterican® 0.45x12 mm) and centrifuged at 12.000 g at 4 °C for 20 min. The flagellar proteins were precipitated from the supernatant using ammonium sulfate (30 %) overnight at room temperature followed by centrifugation at 12.000 g at 4 °C for 20 min. The protein pellet was resuspended in 100 µl Laemmli-buffer[2] and subjected to SDS-PAGE (20 µl sample).

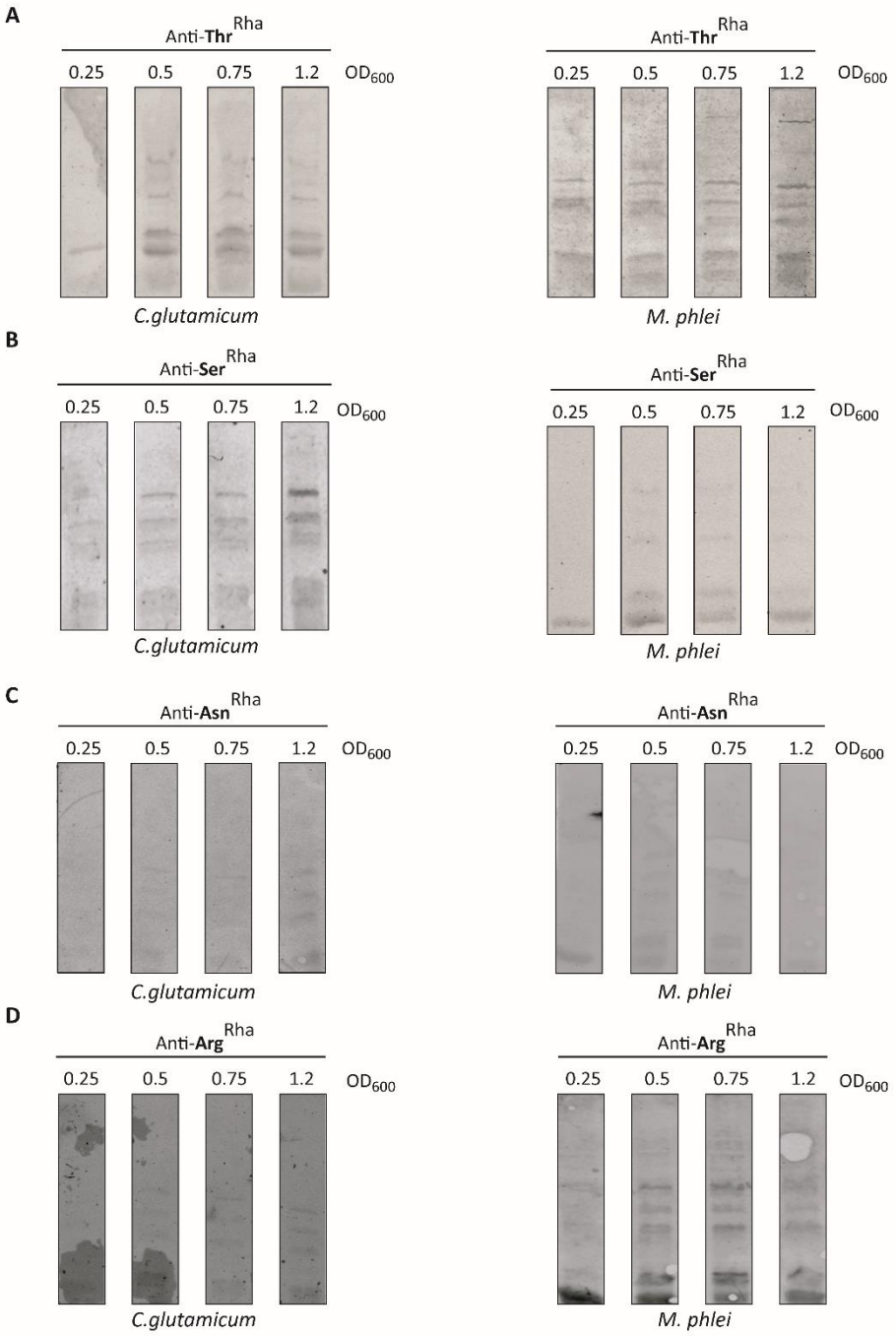


Figure 10: Immunodetection of *Corynebacterium glutamicum* (left) and *Mycobacterium phlei* rhamnoproteins (right) in membrane fractions from different growth phases. Sample were collected at 0.25, 0.5, 0.75 and 1.2 O.D. (600 nm) and subjected to SDS-PAGE with subsequent Western Blot analysis using 0.2 mg/ml of the corresponding antibody: **A**) *anti*-Thr^{Rha}, **B**) *anti*-Ser^{Rha}, **C**) *anti*-Asn^{Rha} and **D**) *anti*-Arg^{Rha}.

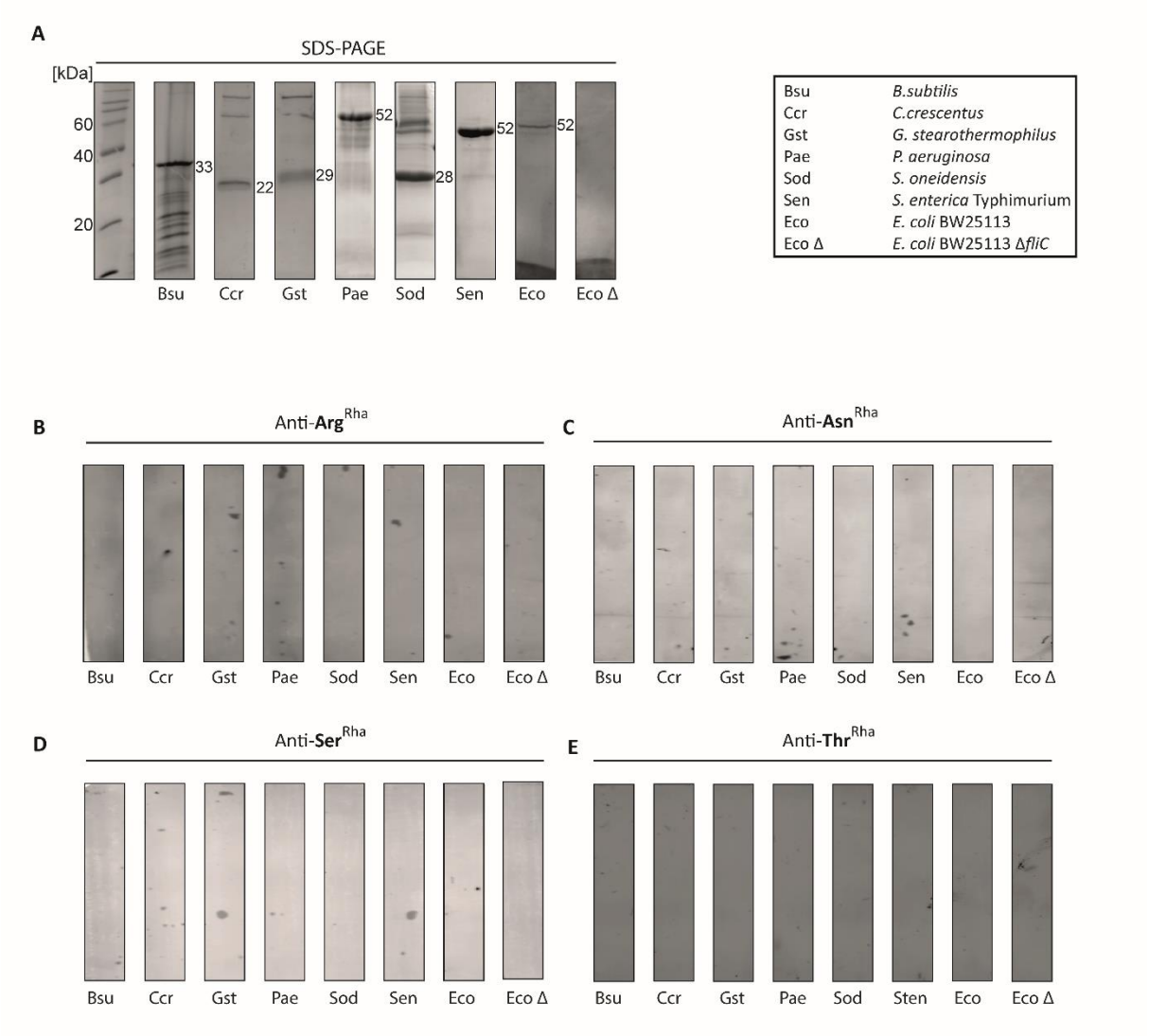
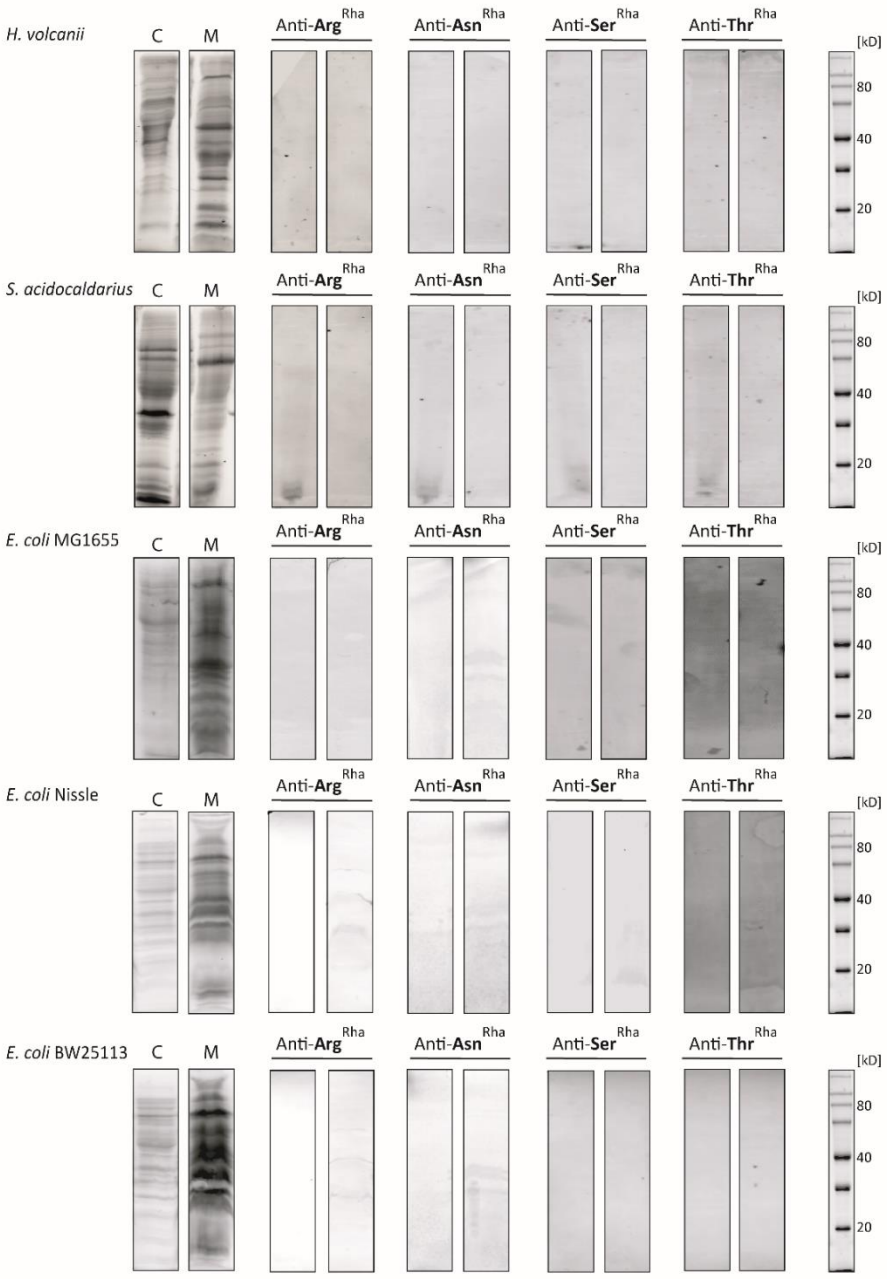
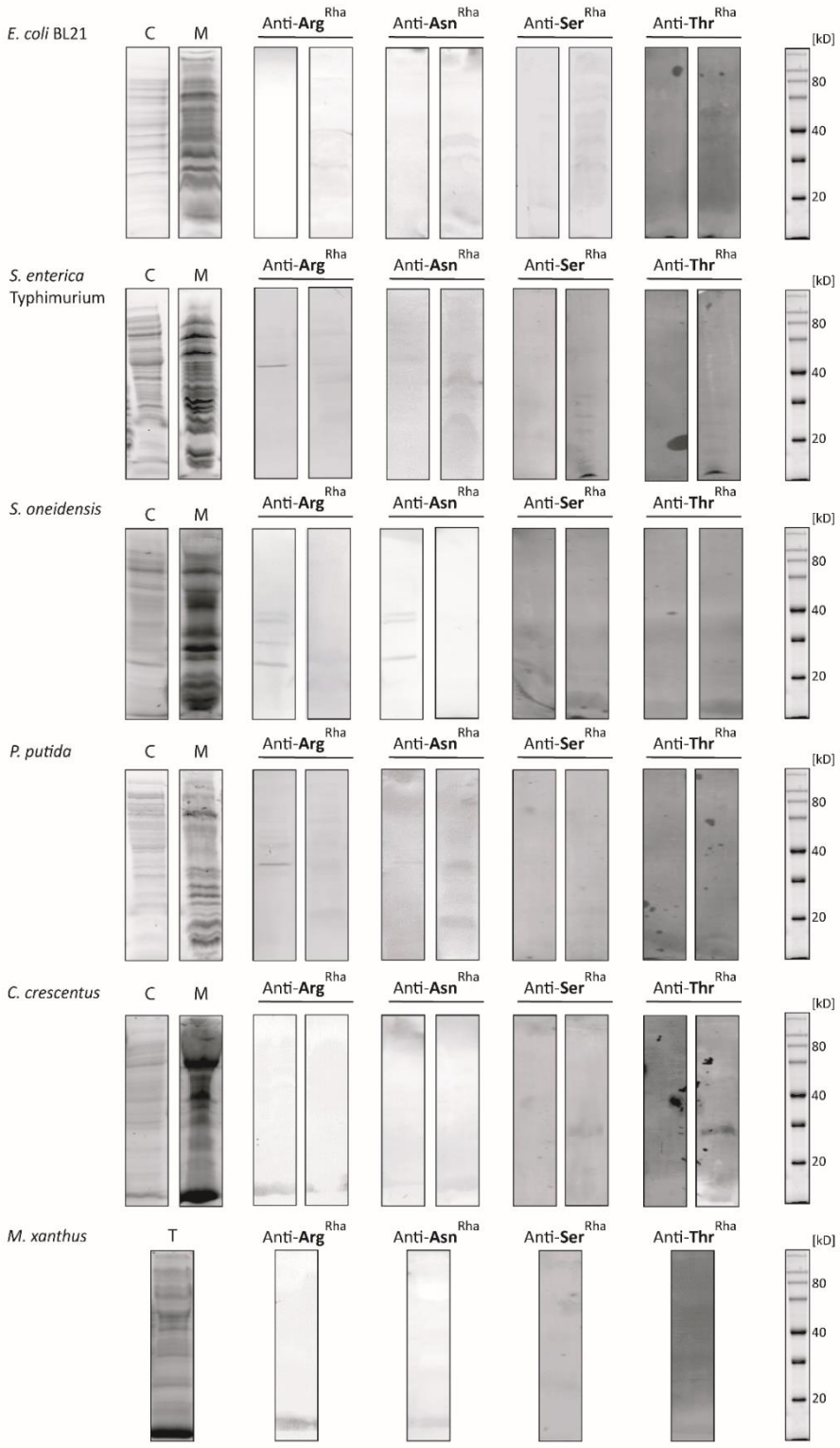


Figure 11: **A**) SDS-PAGE of purified flagellar proteins of swimming bacteria. Flagellar proteins were isolated from overnight cultures using ammonium sulfate precipitation. An *Escherichia coli* strain lacking the flagellar protein FliC (Δ*fliC*) was used as negative control. The expected size of the flagellar proteins is indicated (*Bacillus subtilis*: 33 kDa [34], *Caulobacter crescentus*: 22 kDa [34], *Geobacillus stearothermophilus*: 29 kDa [35], *Pseudomonas aeruginosa*: 52 kDa [36], *Shewanella oneidensis*: 28 kDa [34], *Salmonella enterica* Typhimurium: 52 kDa [34], *E. coli*: 52 kDa [34]). **B**) – **E**) Immunodetection of flagellar proteins with 0.2 mg/ml of the corresponding antibody.


H. volcanii
C
M
Anti-Arg Rha
Anti-Asn Rha
Anti-Ser Rha
Anti-Thr Rha
[kD]
80
40
20
S. acidocaldarius
E. coli MG1655
E. coli Nissle
E. coli BW25113


E. coli BL21
C
M
Anti-Arg Rha
Anti-Asn Rha
Anti-Ser Rha
Anti-Thr Rha
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20
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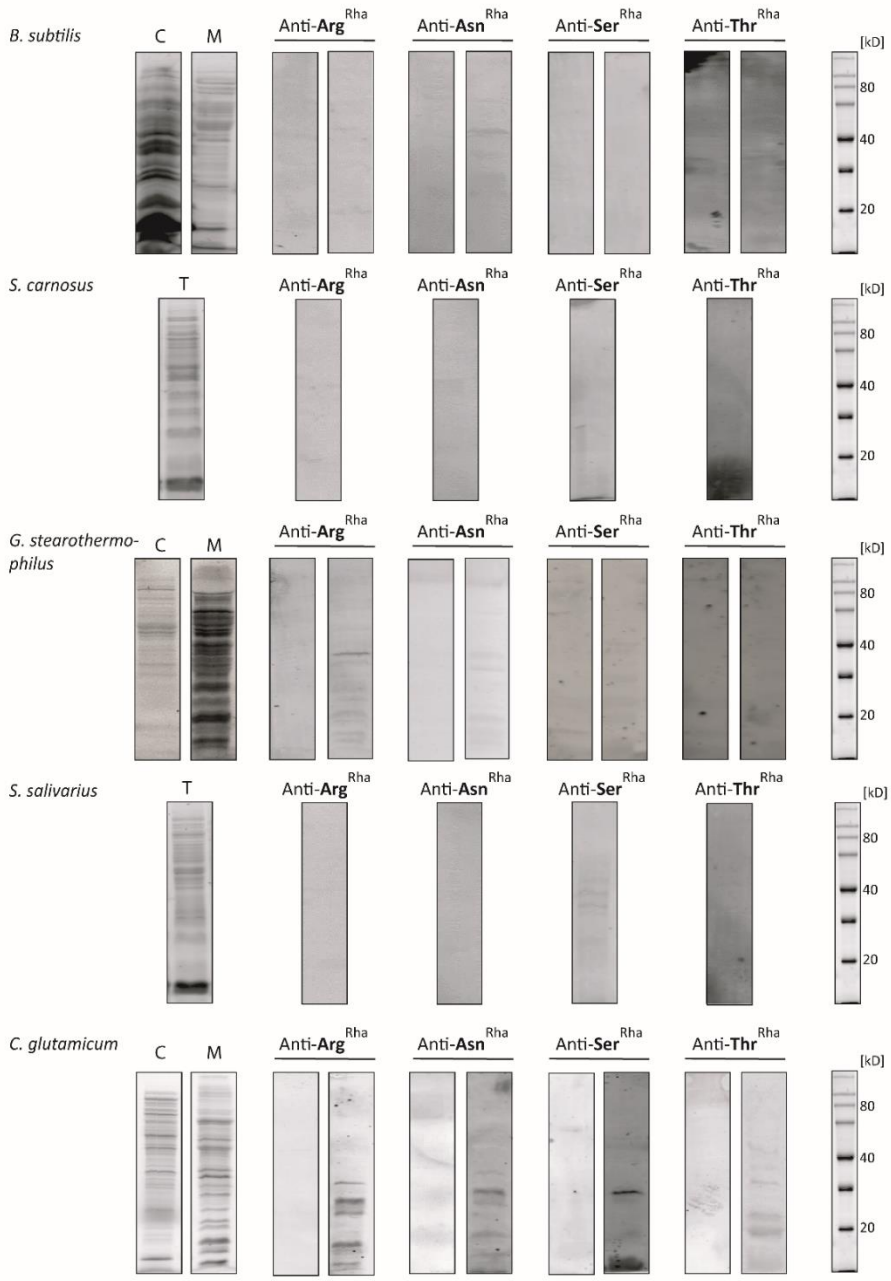
P. putida
C. crescentus
M. xanthus
T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S. salivarius
C. glutamicum

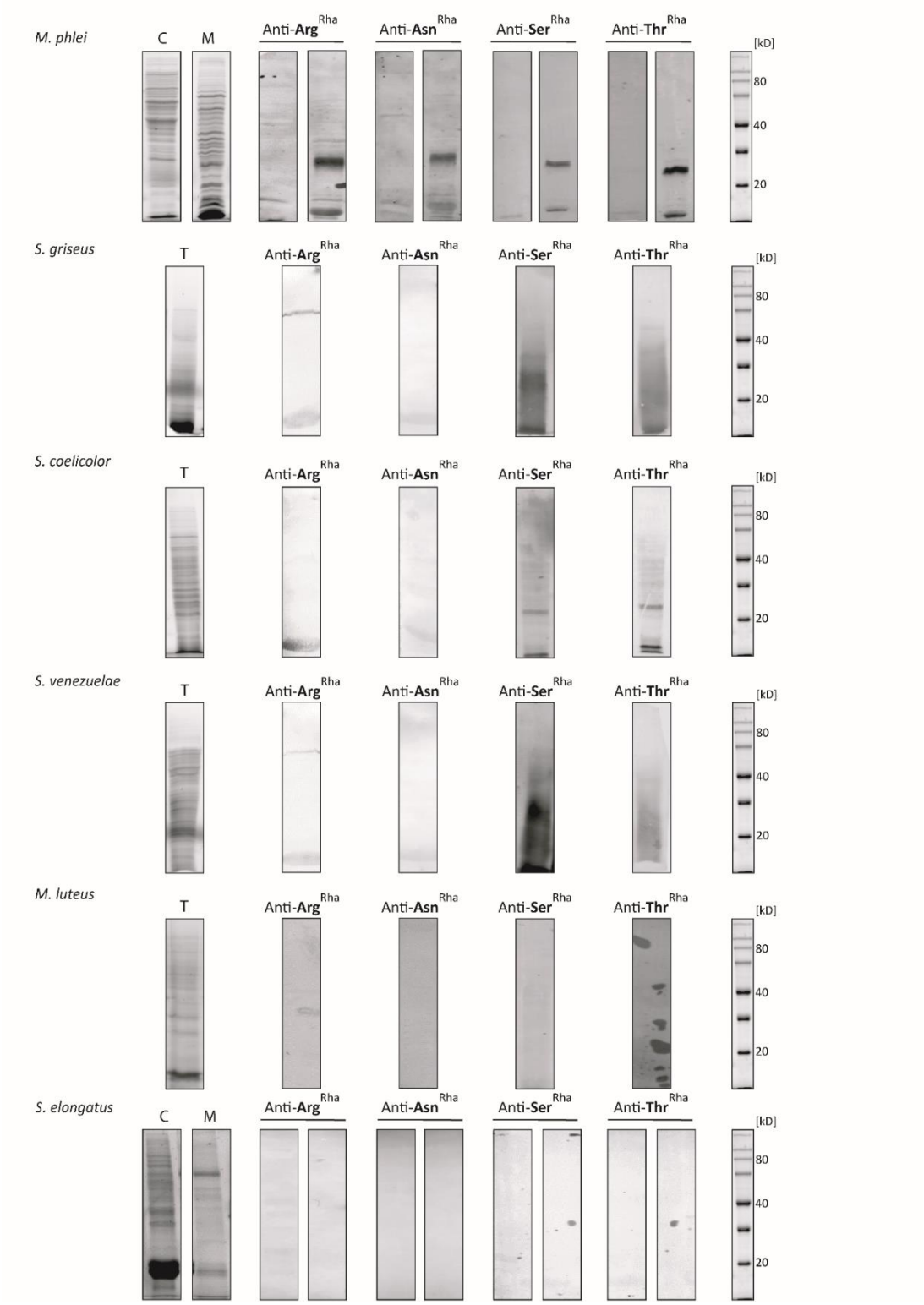
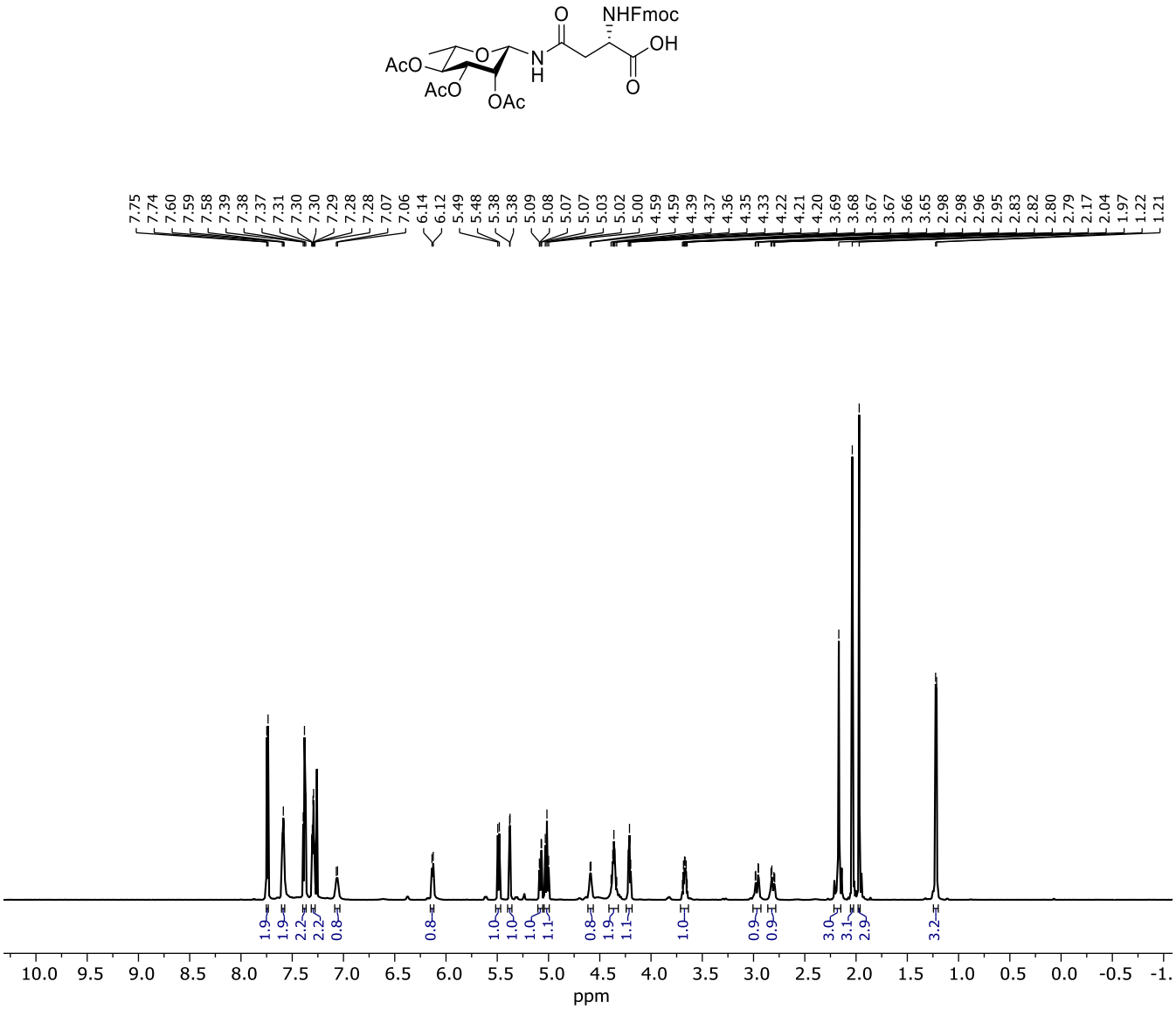


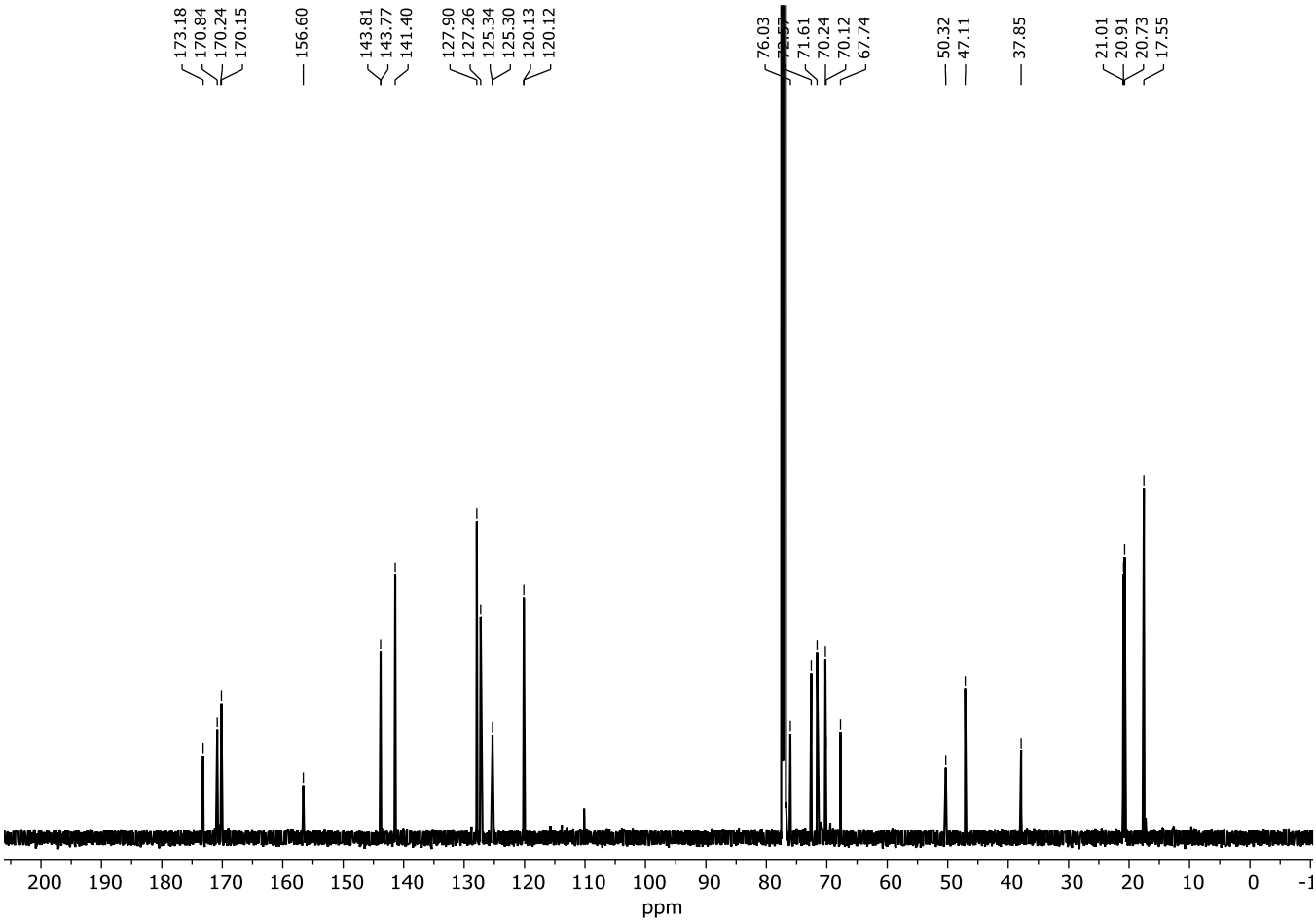
Figure 12: Immunodetection of rhamnosylated proteins using *anti*-Arg^{Rha}, *anti*-Asn^{Rha}, *anti*-Ser^{Rha} and *anti*-Thr^{Rha}. Prokaryotic samples were lysed (T) where indicated separated into cytosolic (C) and membrane (M) fractions. Total protein was visualized from SDS-PAGE (left) size separated proteins using 2,2,2-trichlorethanol. A subsequent Western Blot analysis with 0.2 mg/ml of the respective aa^{Rha} specific antibody is shown on the right.

NMR Spectra and HPLC Chromatograms

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-β-L-rhamnosyl)-L-asparagine (**7**)

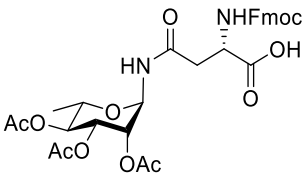


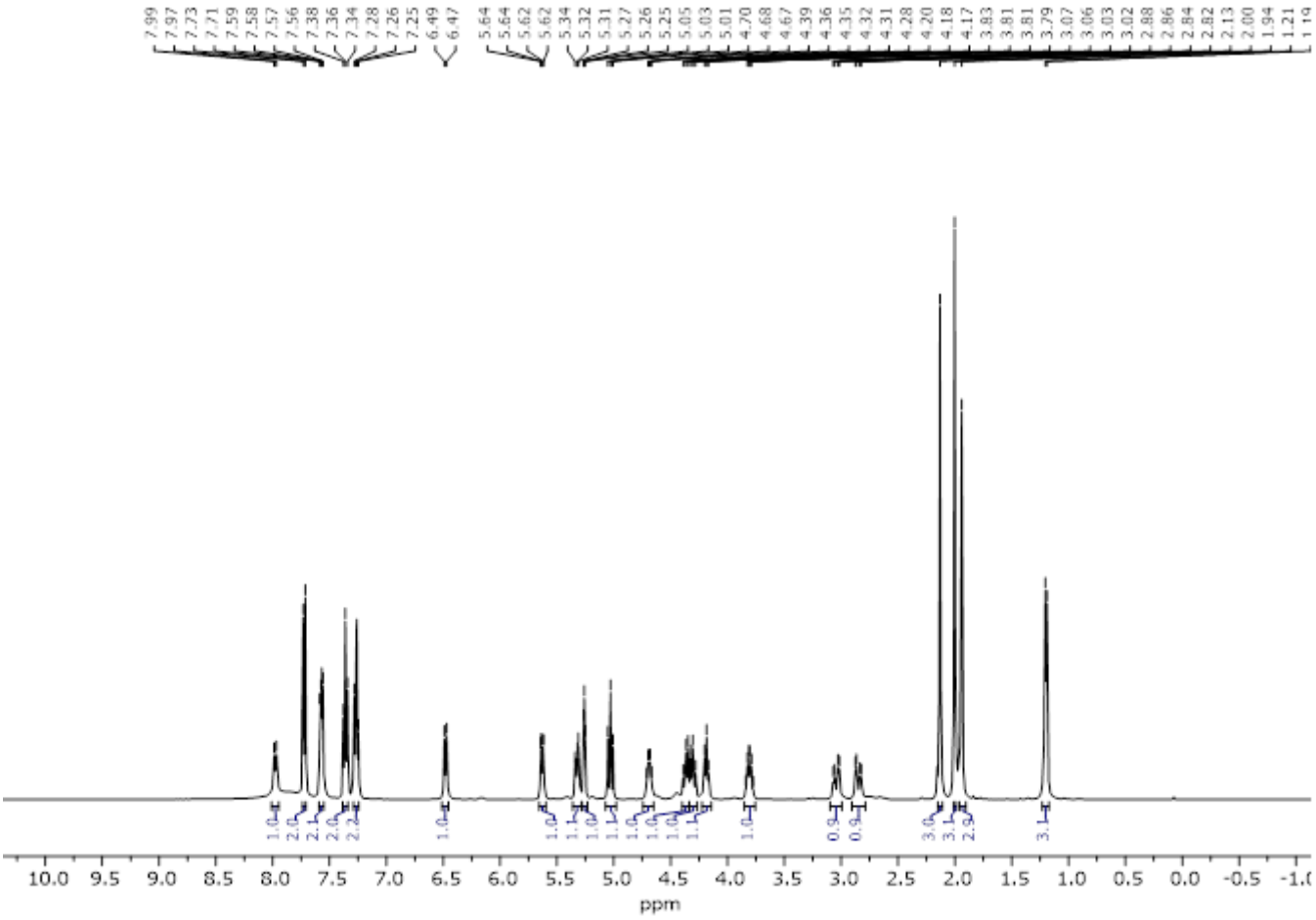
^{1}H-NMR Spectrum of **7** ($CDCl_3$, 600 MHz)



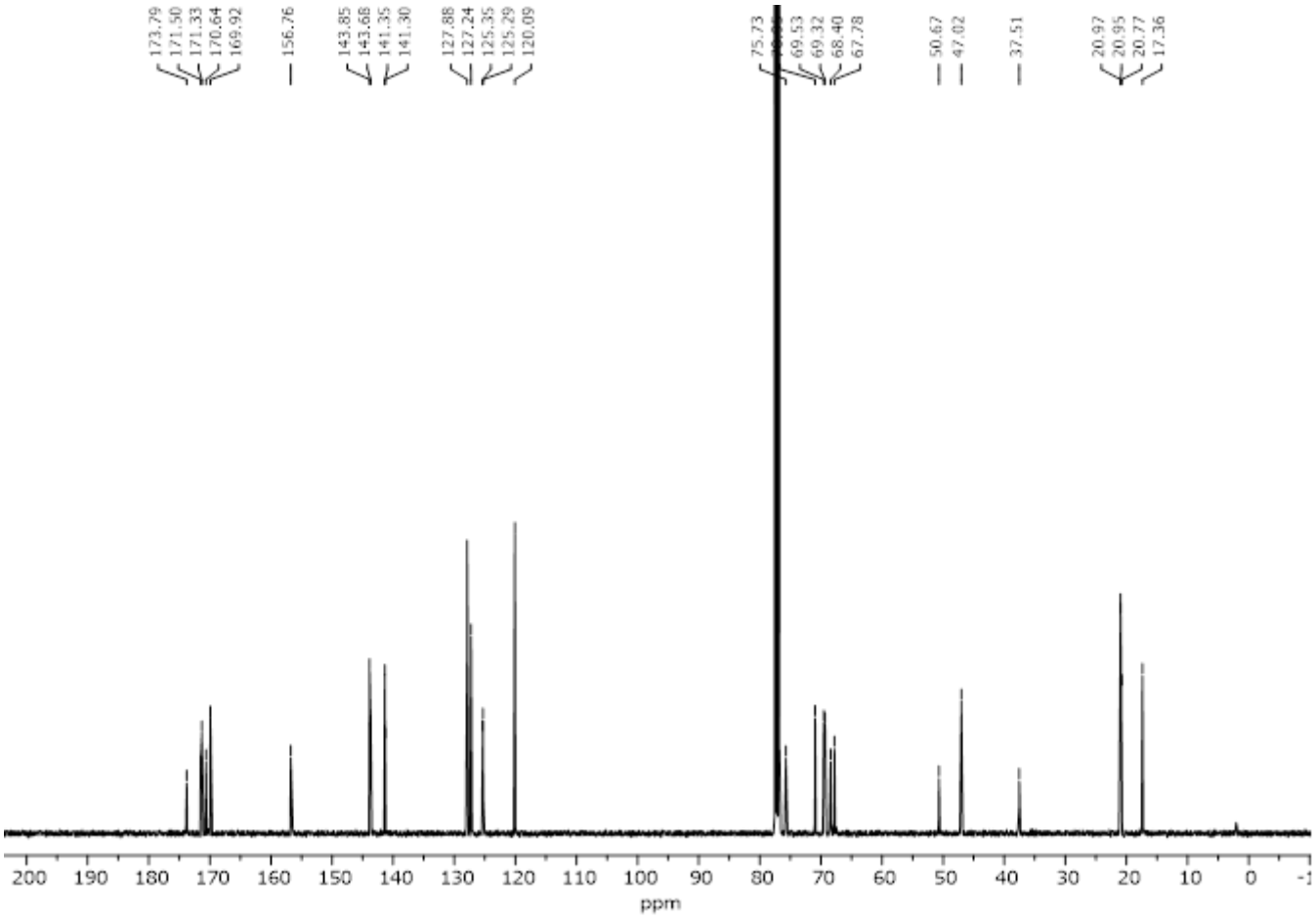
^{13}C-NMR Spectrum of **7** ($CDCl_3$, 150 MHz)

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*N*$^{\gamma}$-(2,3,4-Tri-*O*-acetyl-α-L-rhamnosyl)-L-asparagine (**9**)



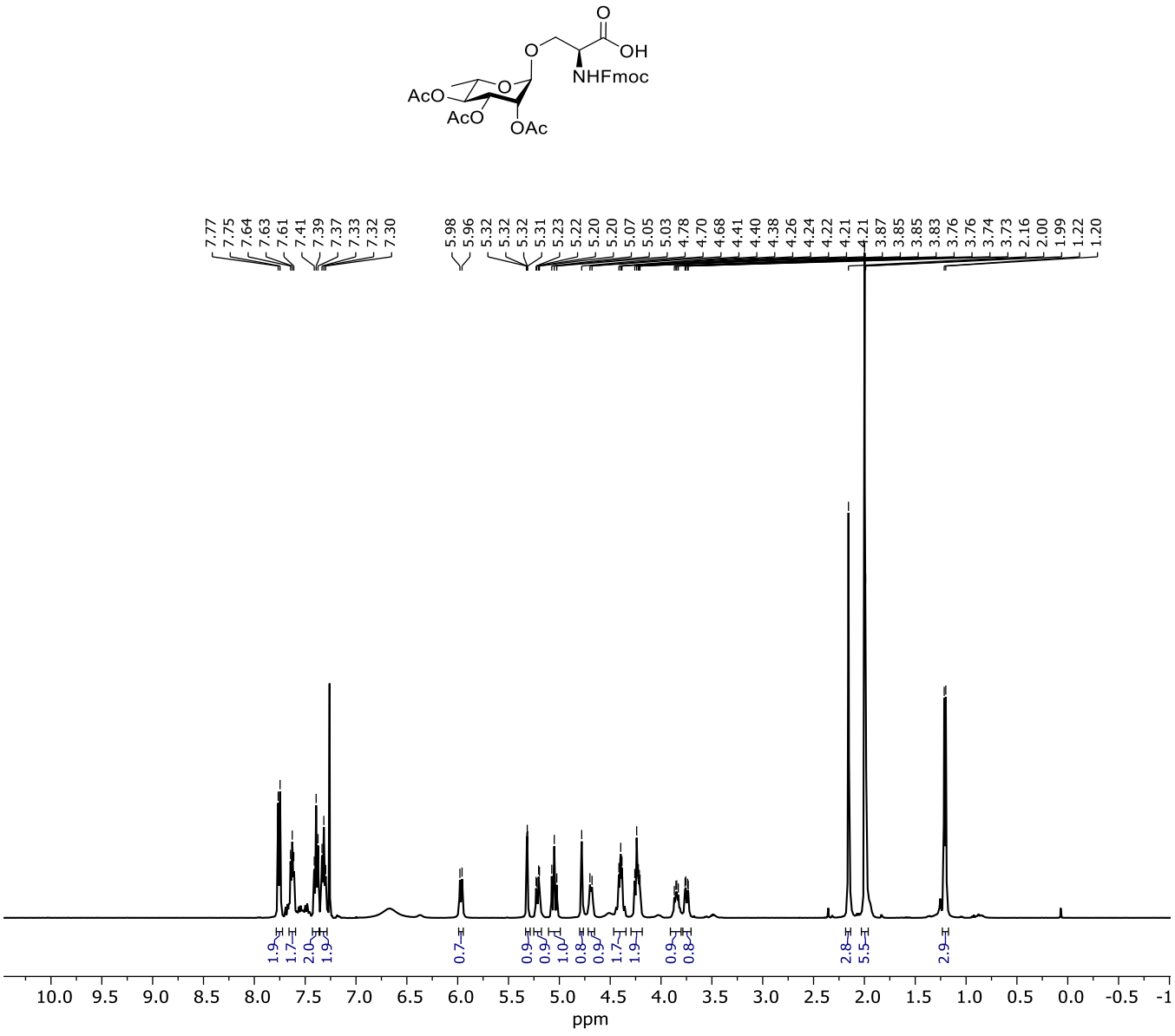


^{1}H-NMR Spectrum of **9** ($CDCl_3$, 400 MHz)

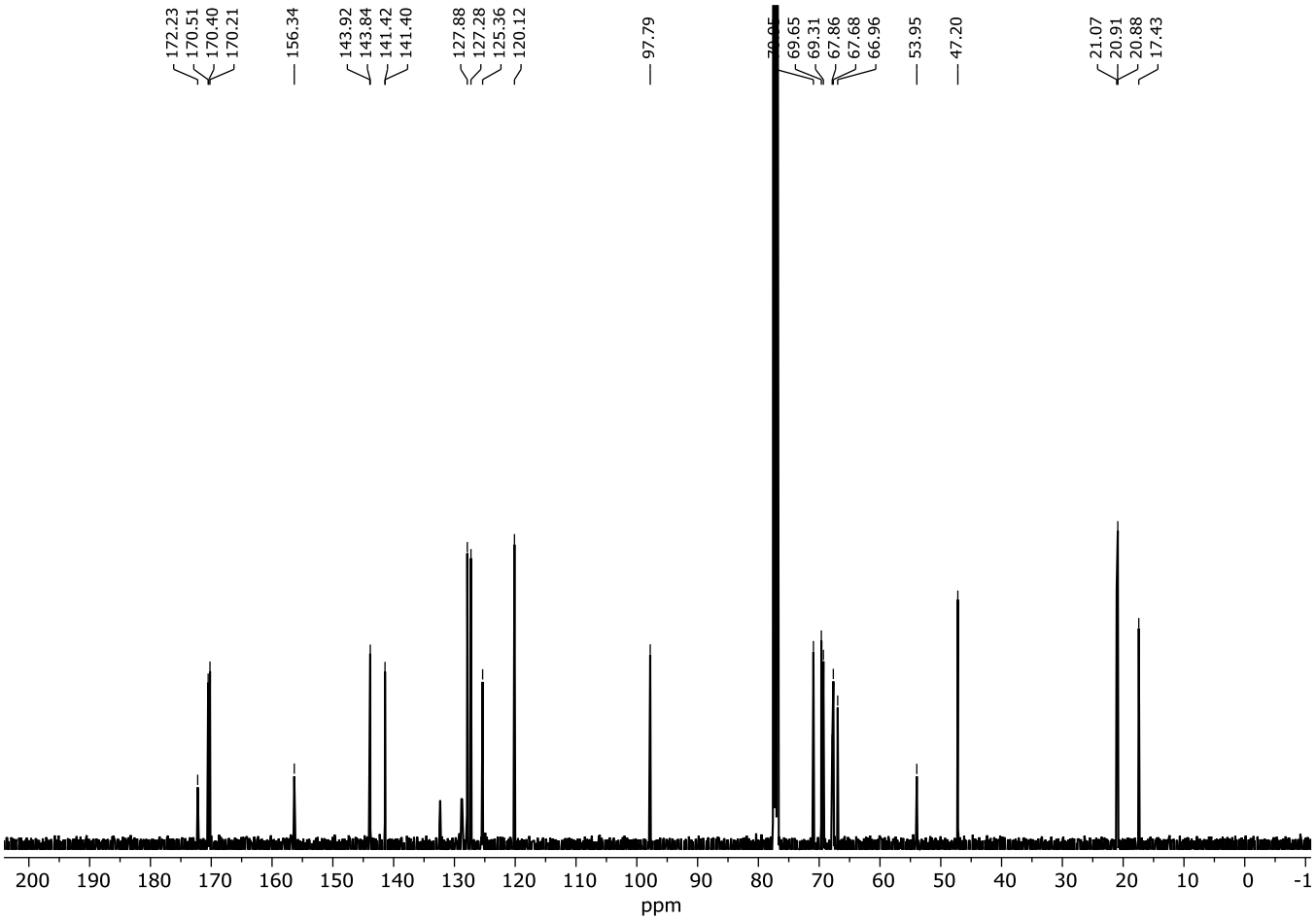


^{13}C-NMR Spectrum of **9** ($CDCl_3$, 100 MHz)

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-serine (**1**)

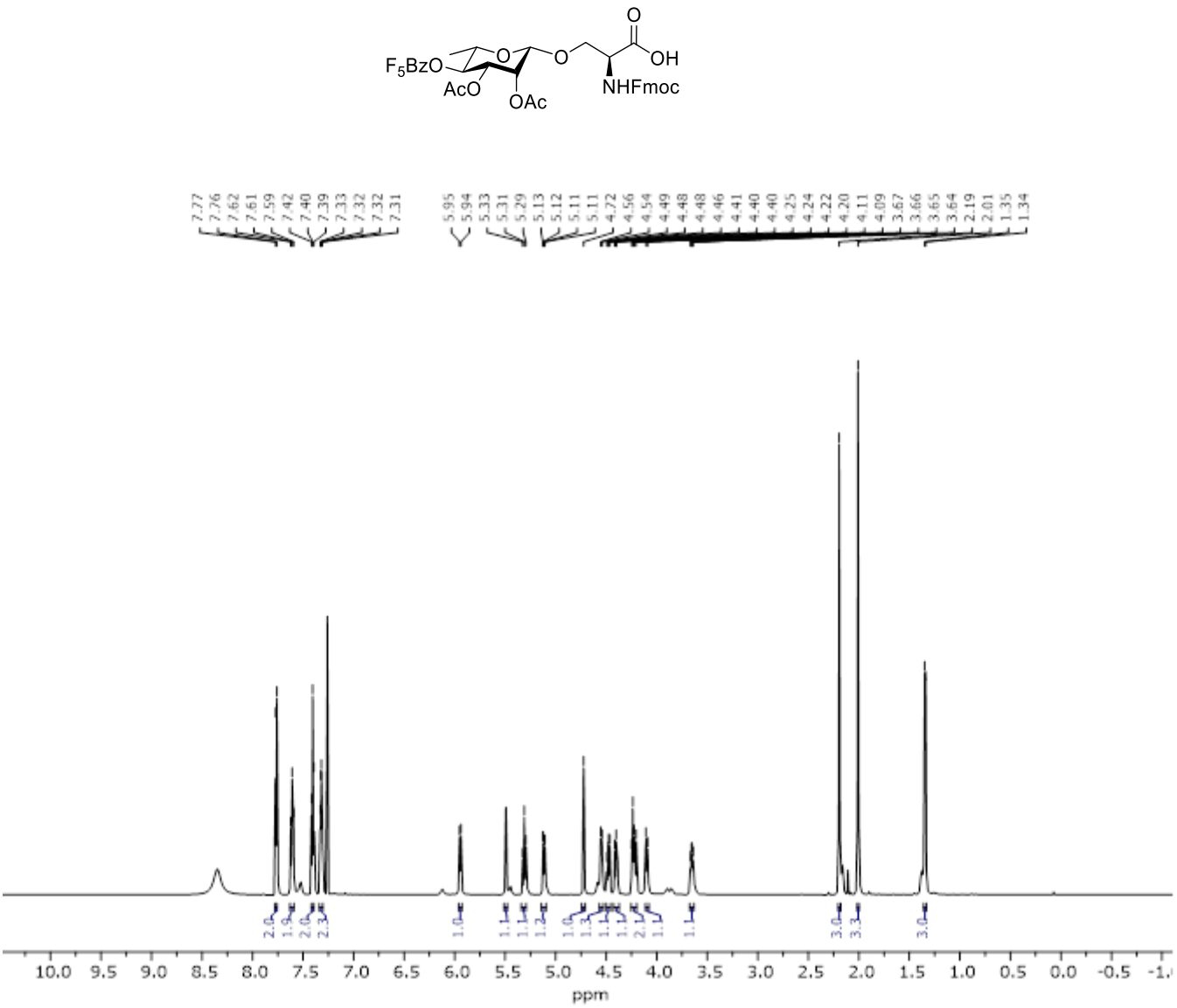


^{1}H-NMR Spectrum of **1** ($CDCl_3$, 400 MHz)

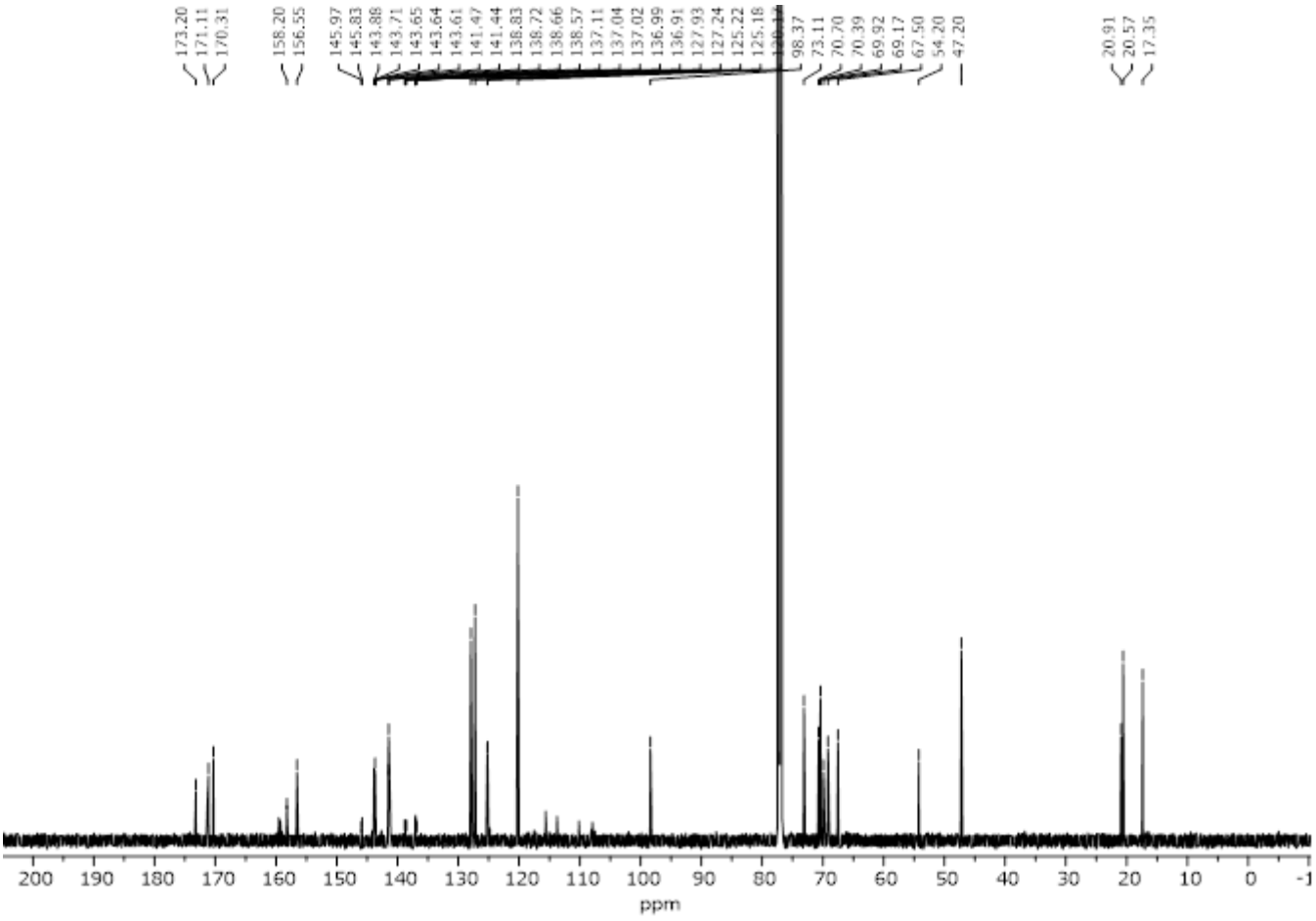


^{13}C-NMR Spectrum of **1** ($CDCl_3$, 100 MHz)

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamnopyranosyl)-L-serine (**4**)

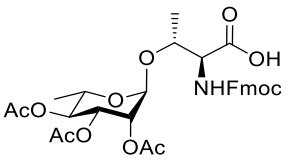


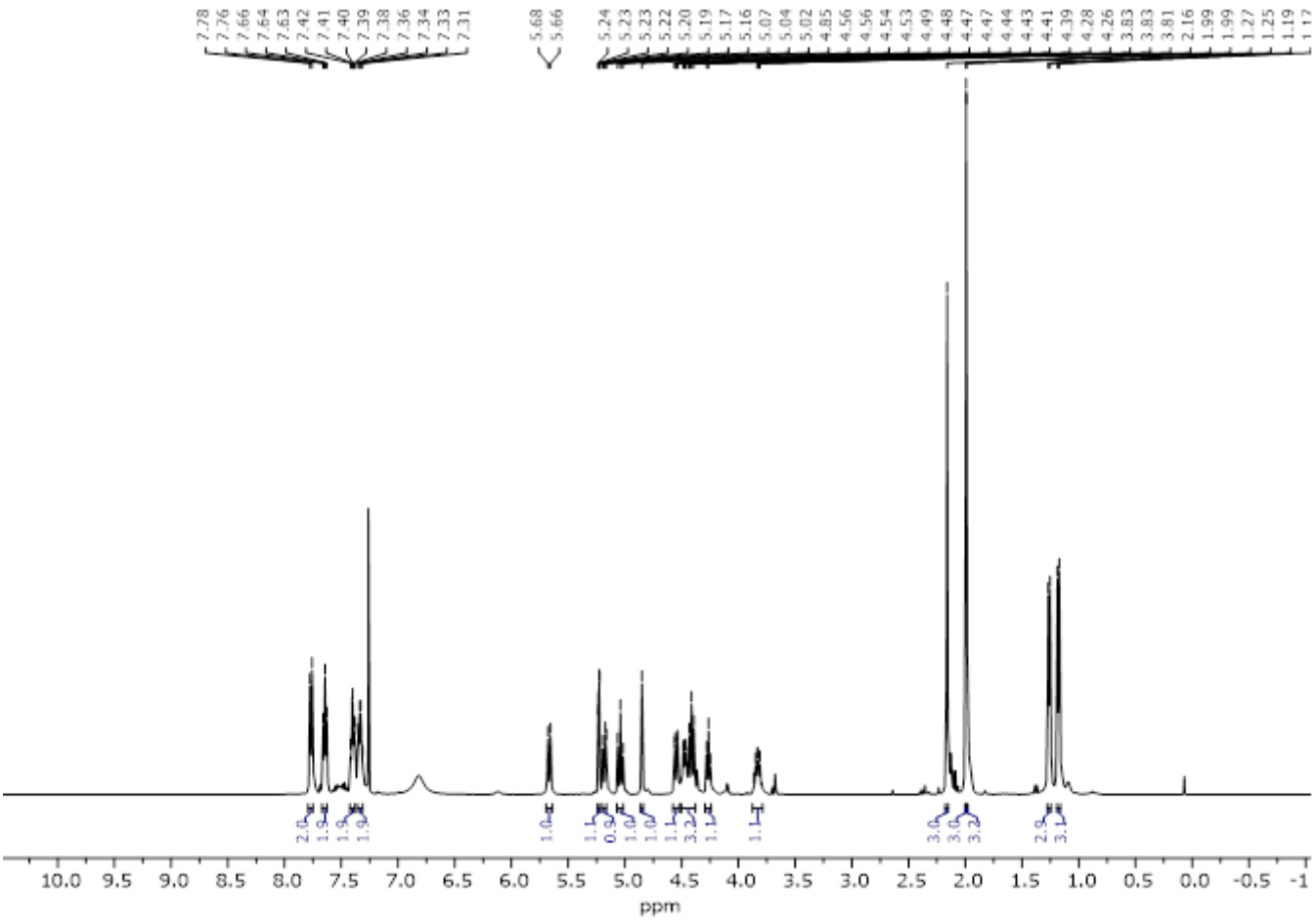
^{1}H-NMR Spectrum of **4** ($CDCl_3$, 600 MHz)



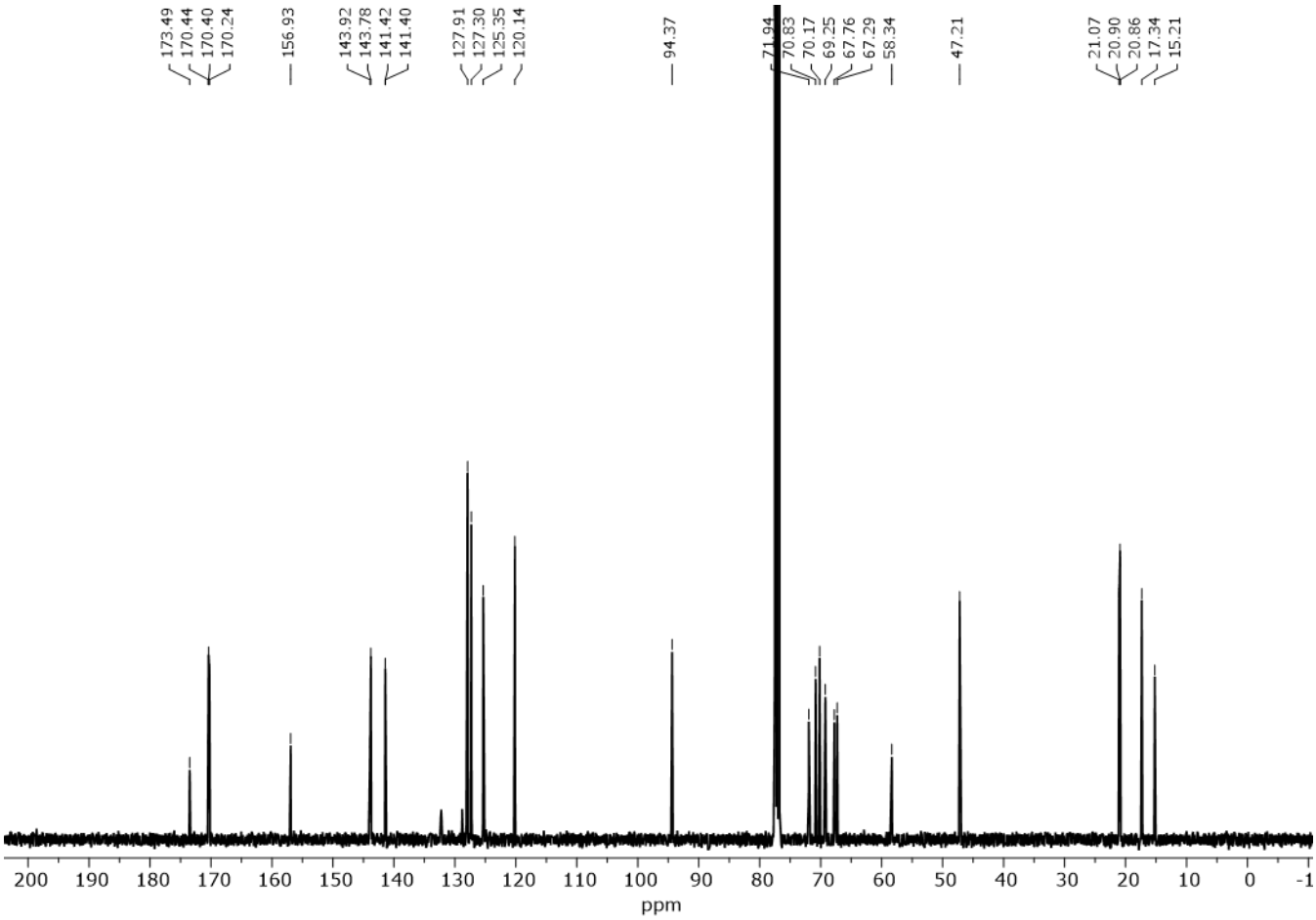
^{13}C-NMR Spectrum of **4** ($CDCl_3$, 150 MHz)

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-threonine (**2**)



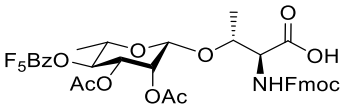


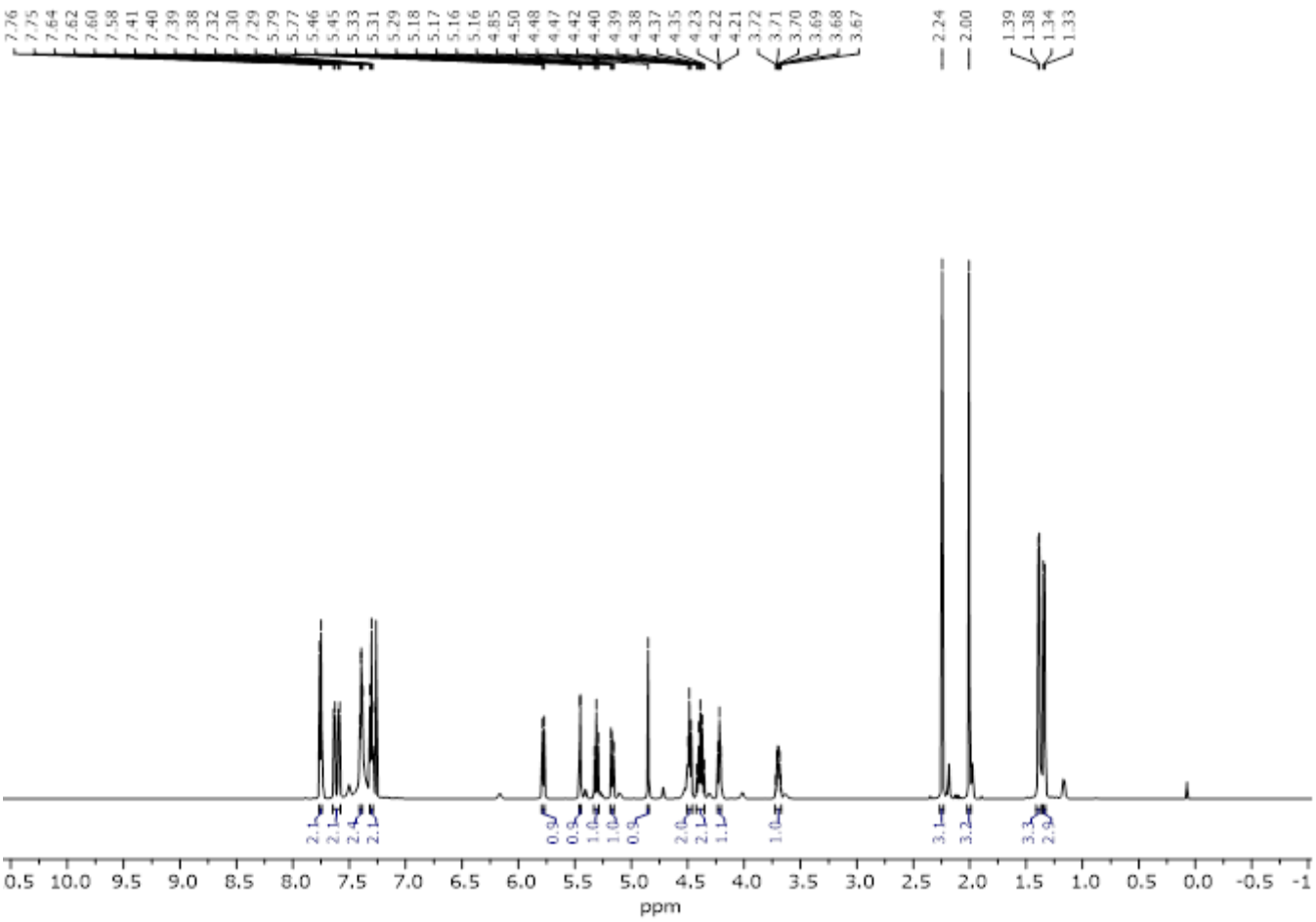
^{1}H-NMR Spectrum of **2** ($CDCl_3$, 400 MHz)



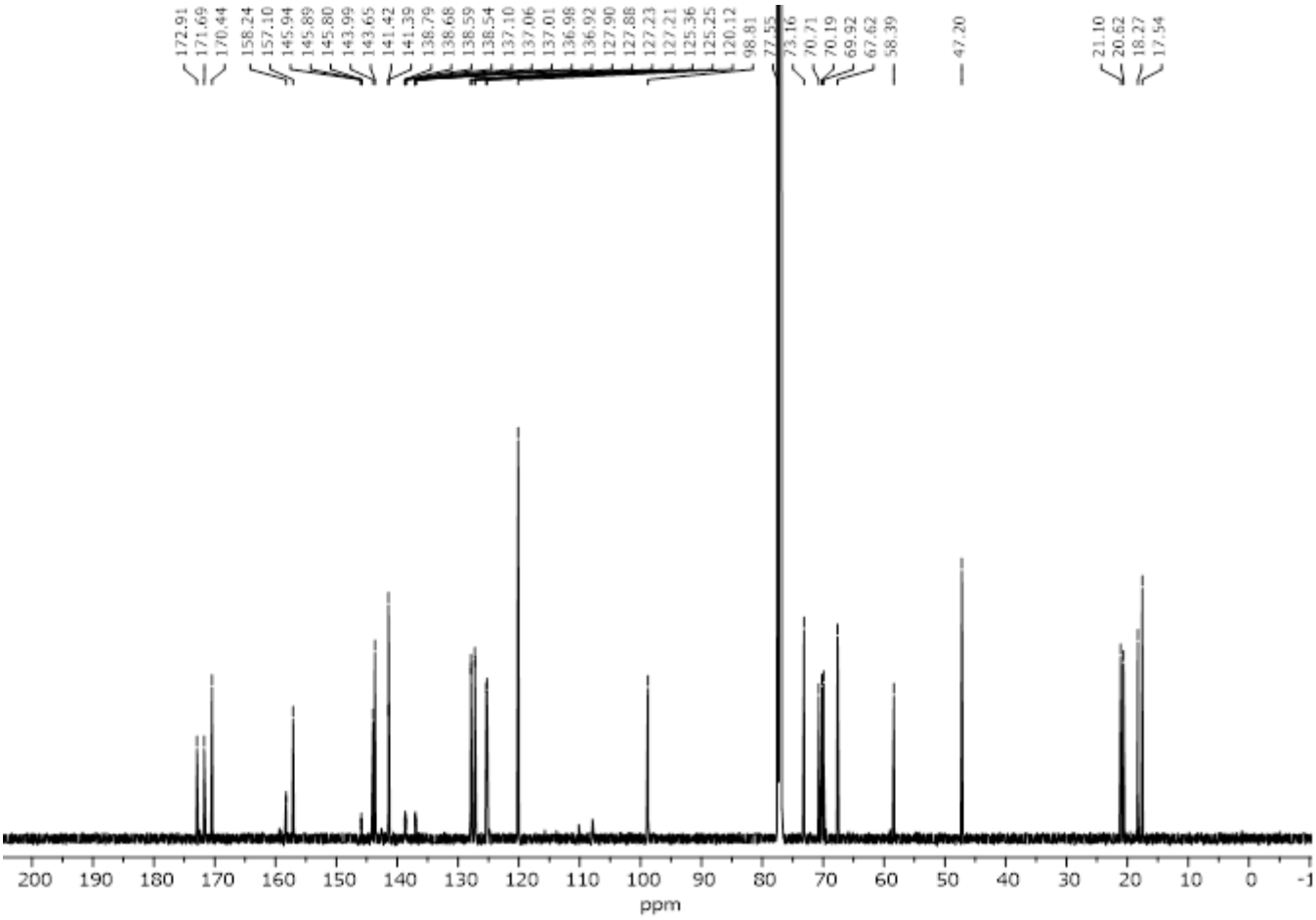
^{13}C-NMR Spectrum of **2** ($CDCl_3$, 100 MHz)

N$^{\alpha}$-Fluorenylmethoxycarbonyl-*O*-(2,3-*O*-acetyl-4-*O*-pentafluorobenzoyl-β-L-rhamnopyranosyl)-L-threonine (**5**)



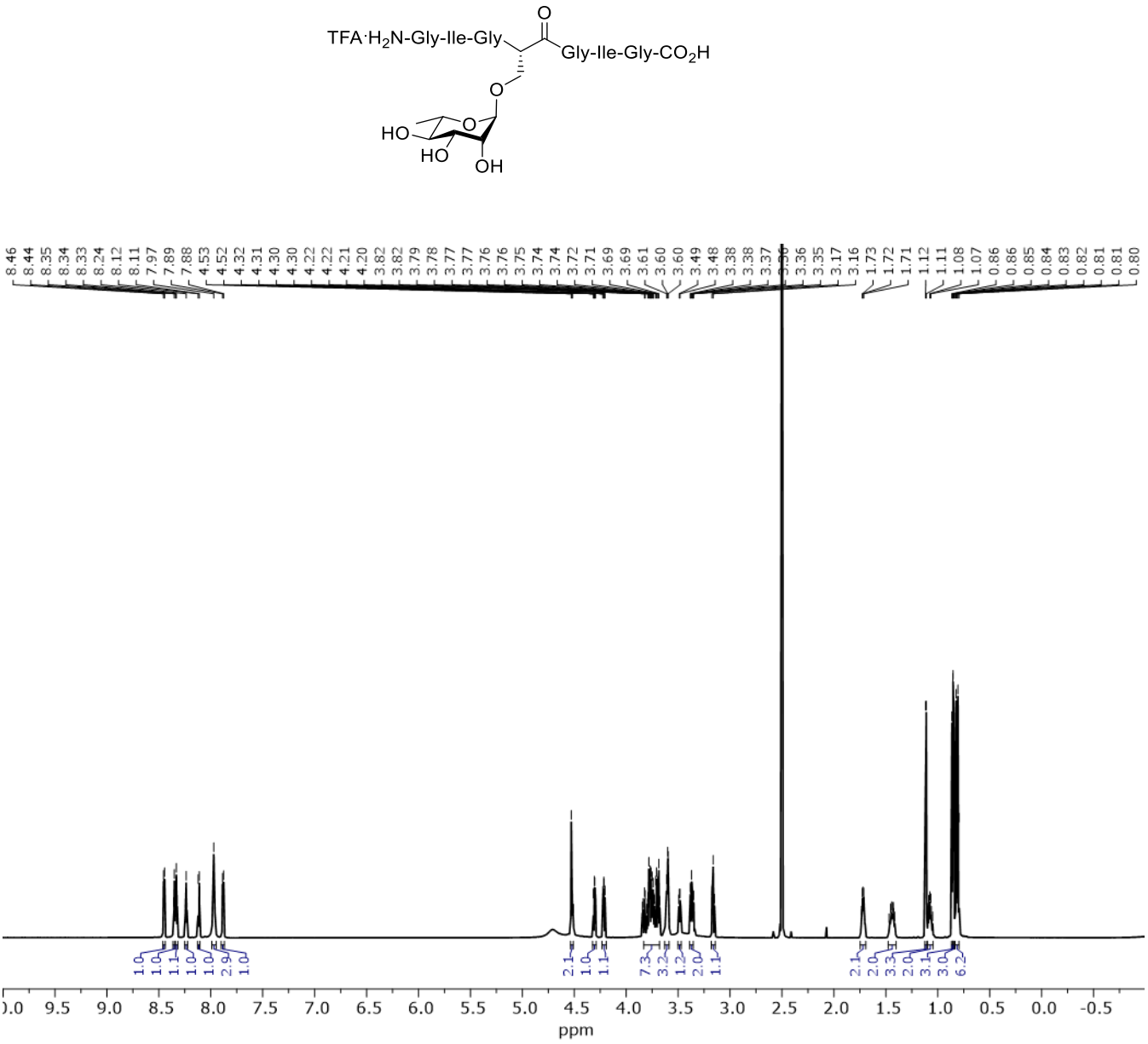


^{1}H-NMR Spectrum of **5** ($CDCl_3$, 600 MHz)

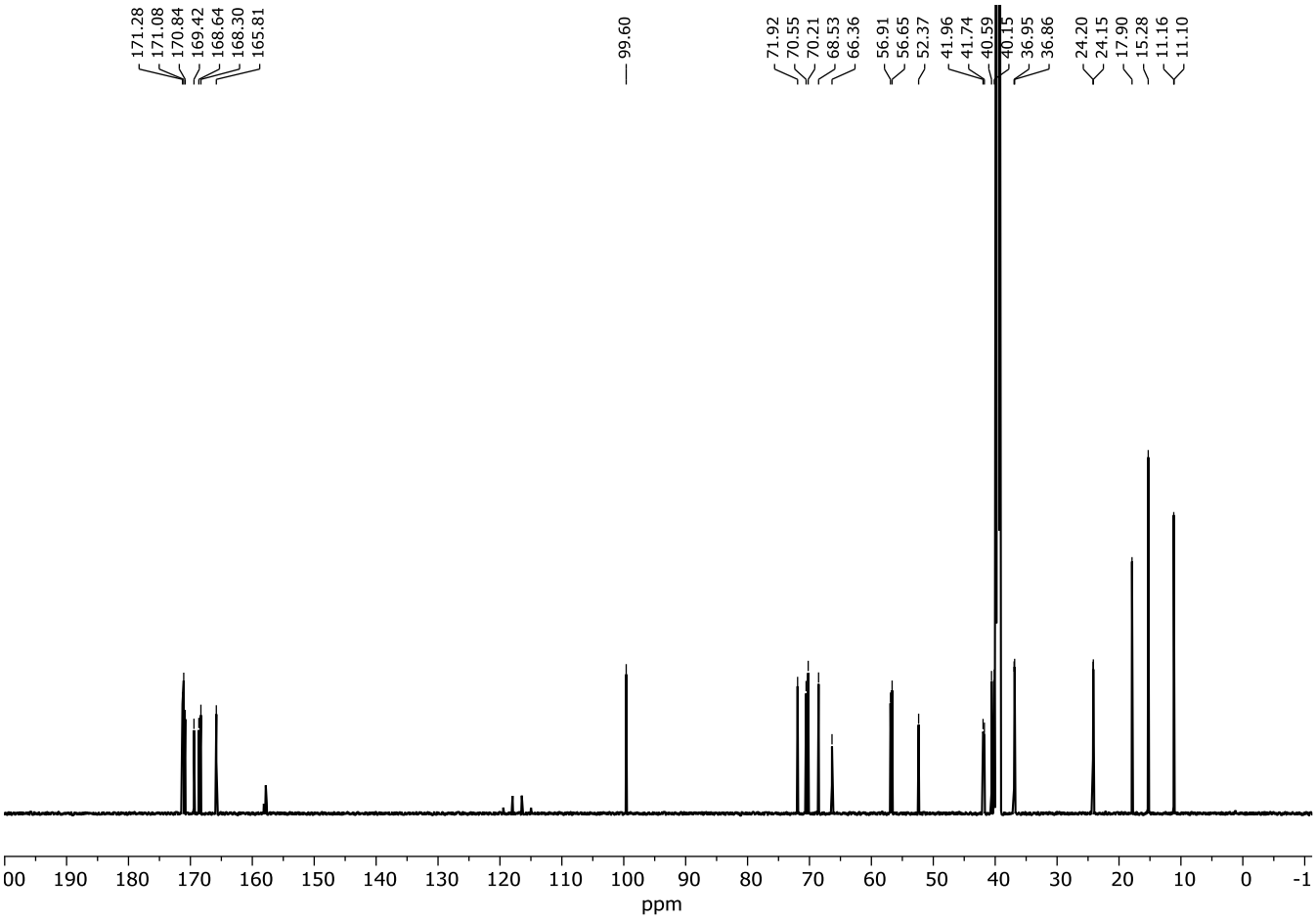


^{13}C-NMR Spectrum of **5** ($CDCl_3$, 150 MHz)

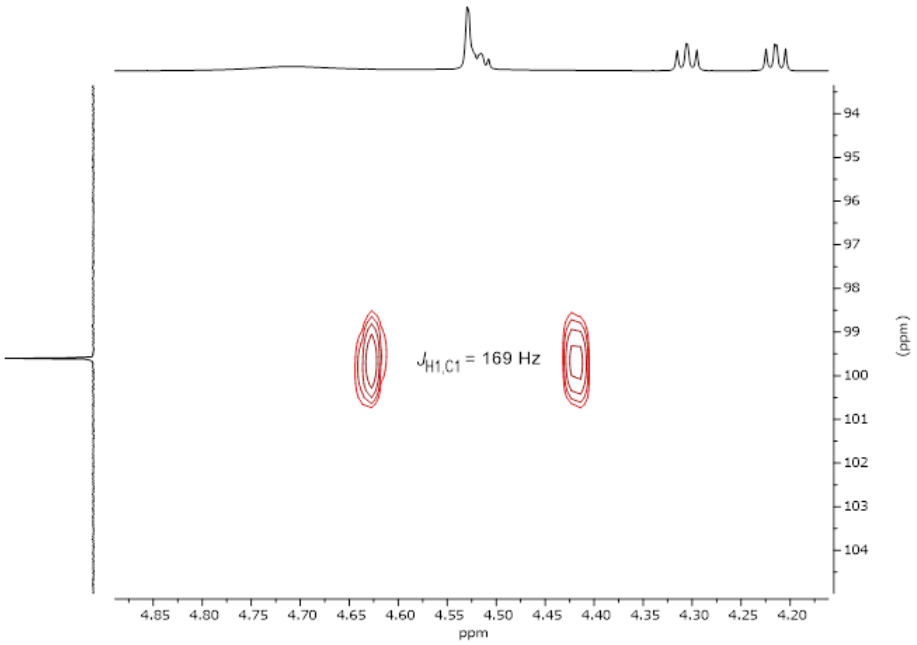
Ser$^{\alpha\text{-Rha}}$-Peptide **11**



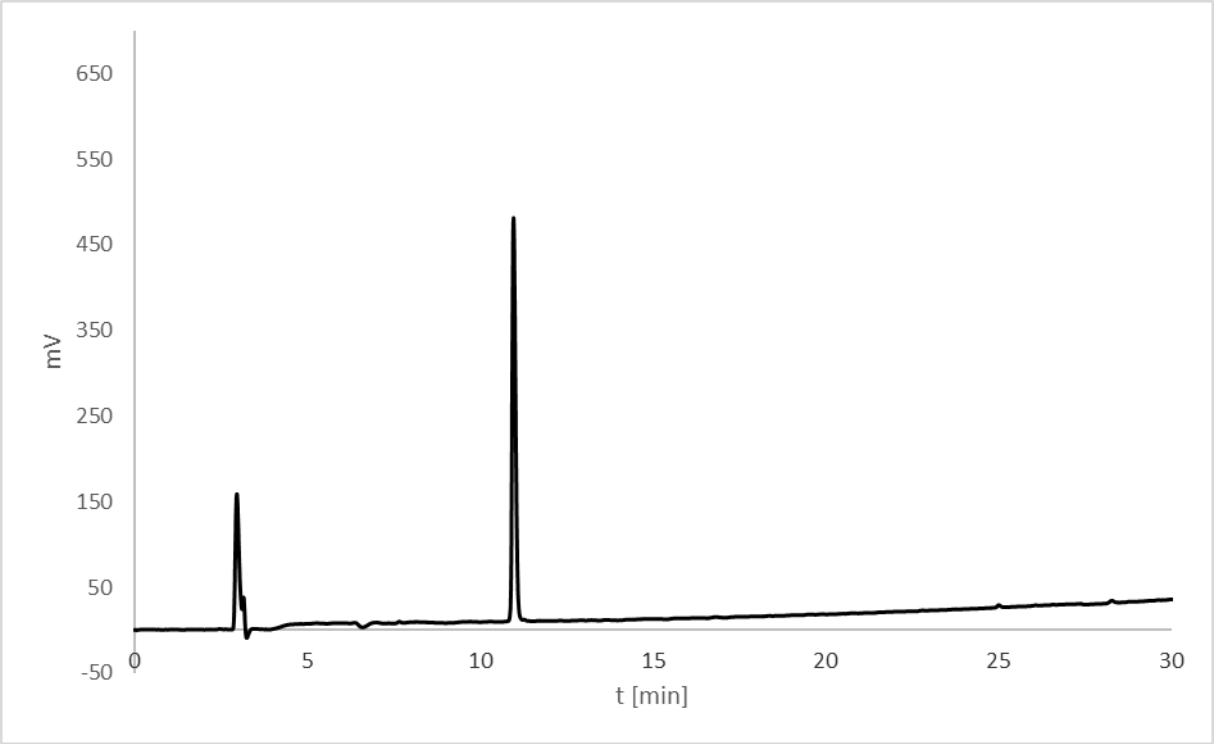
^{1}H-NMR Spectrum of Ser$^{\alpha\text{-Rha}}$-Peptide **11** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Ser$^{\alpha\text{-Rha}}$-Peptide **11** (200 MHz, [d6]-DMSO)

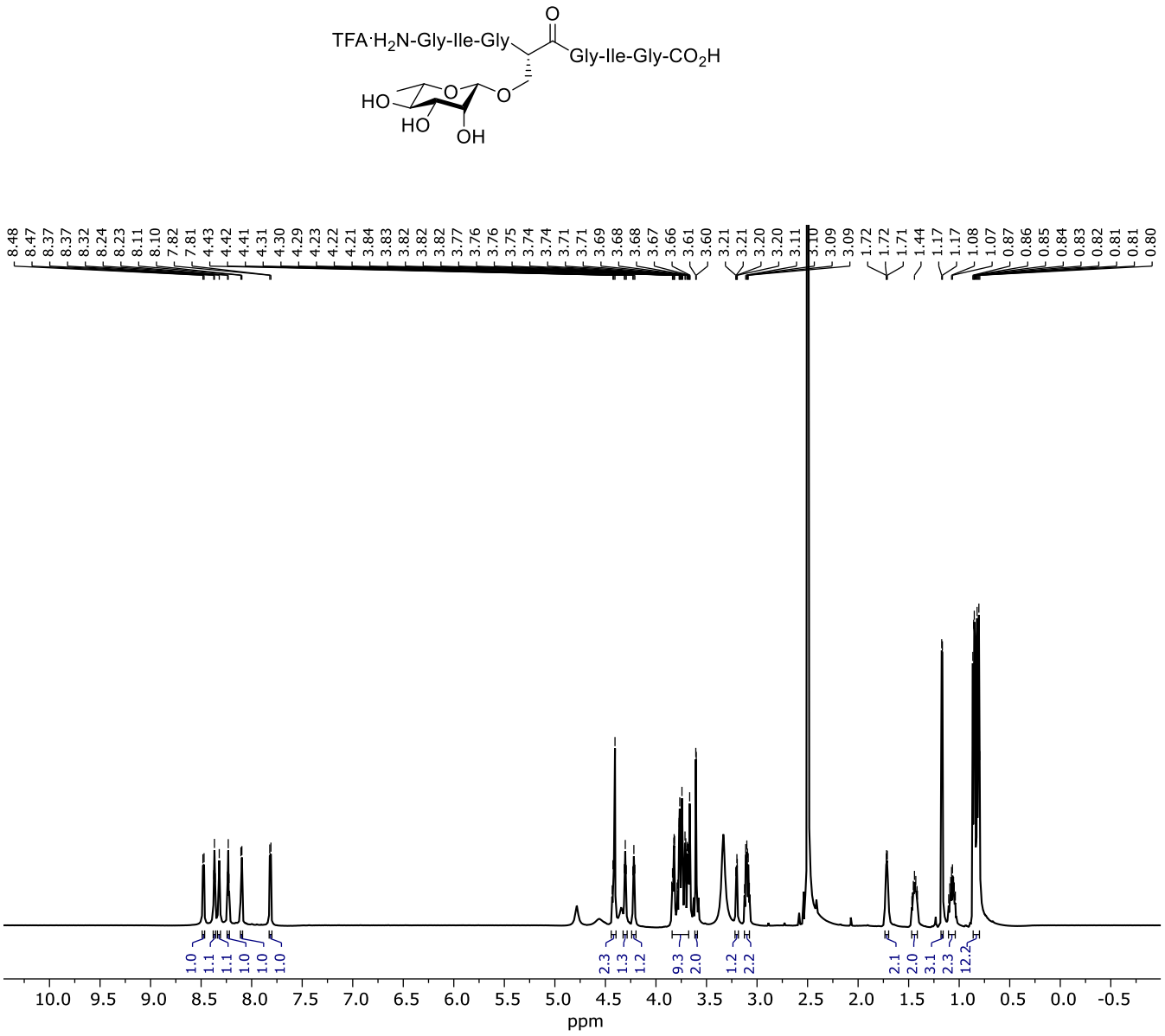


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **11**: H-1-C-1 coupling constant $J_{H1,C1}$

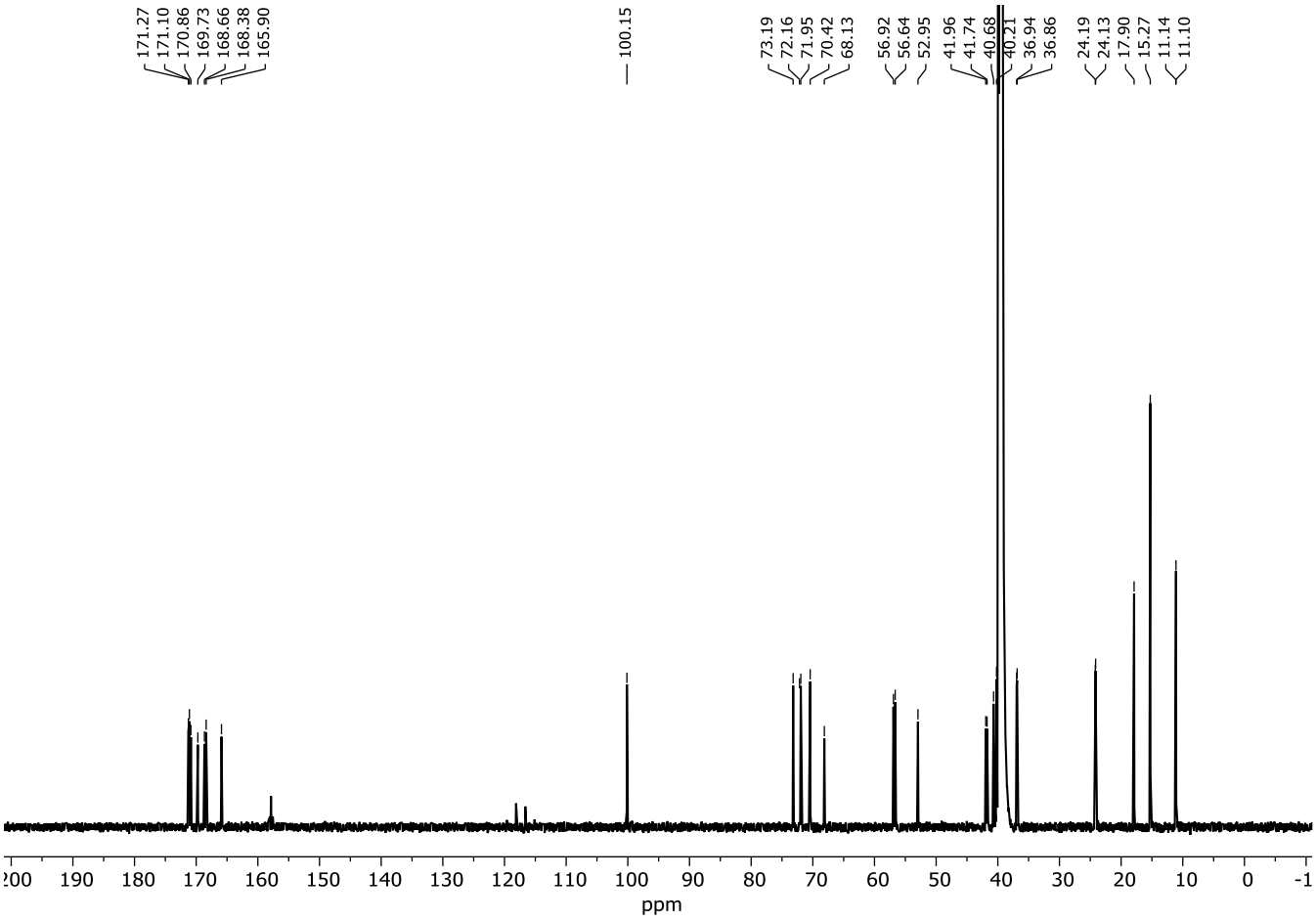


Analytical HPLC-trace of Ser$^{\alpha\text{-Rha}}$-Peptide **11**

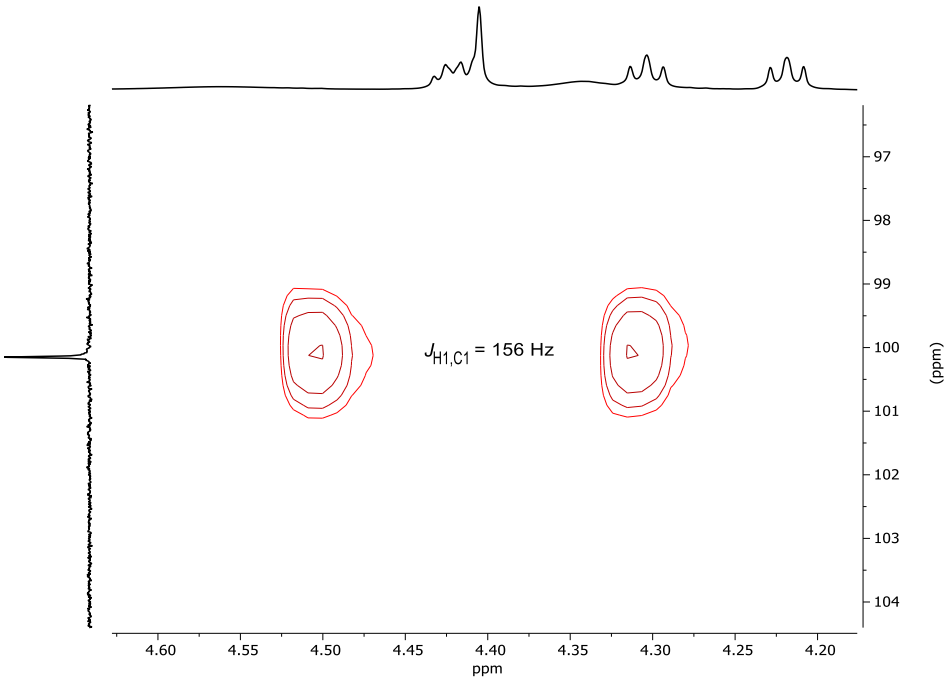
Ser$^{\beta\text{-Rha}}$-Peptide **12**



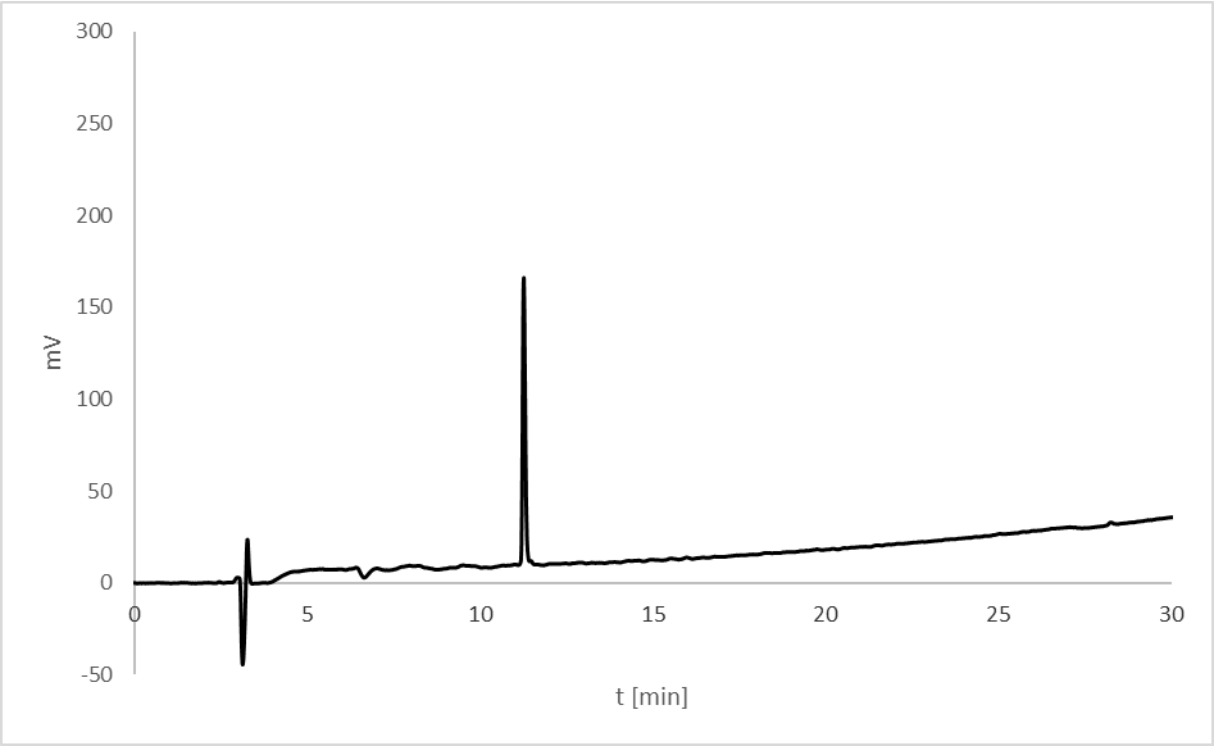
^{1}H-NMR Spectrum of Ser$^{\beta\text{-Rha}}$-Peptide **12** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Ser$^{\beta\text{-Rha}}$-Peptide **12** (200 MHz, [d6]-DMSO)

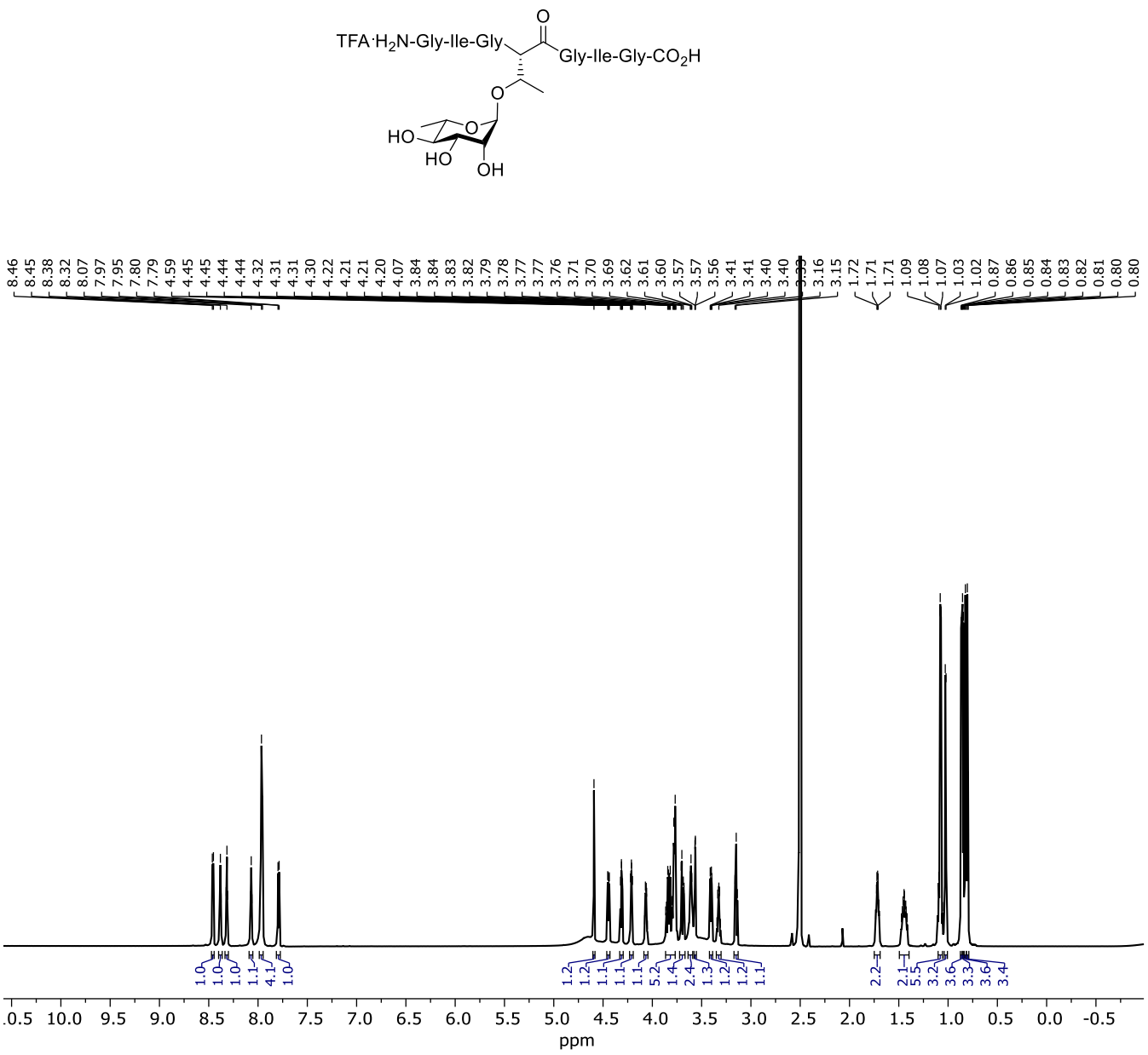


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **12**: H-1,C-1 coupling constant $J_{H1,C1}$

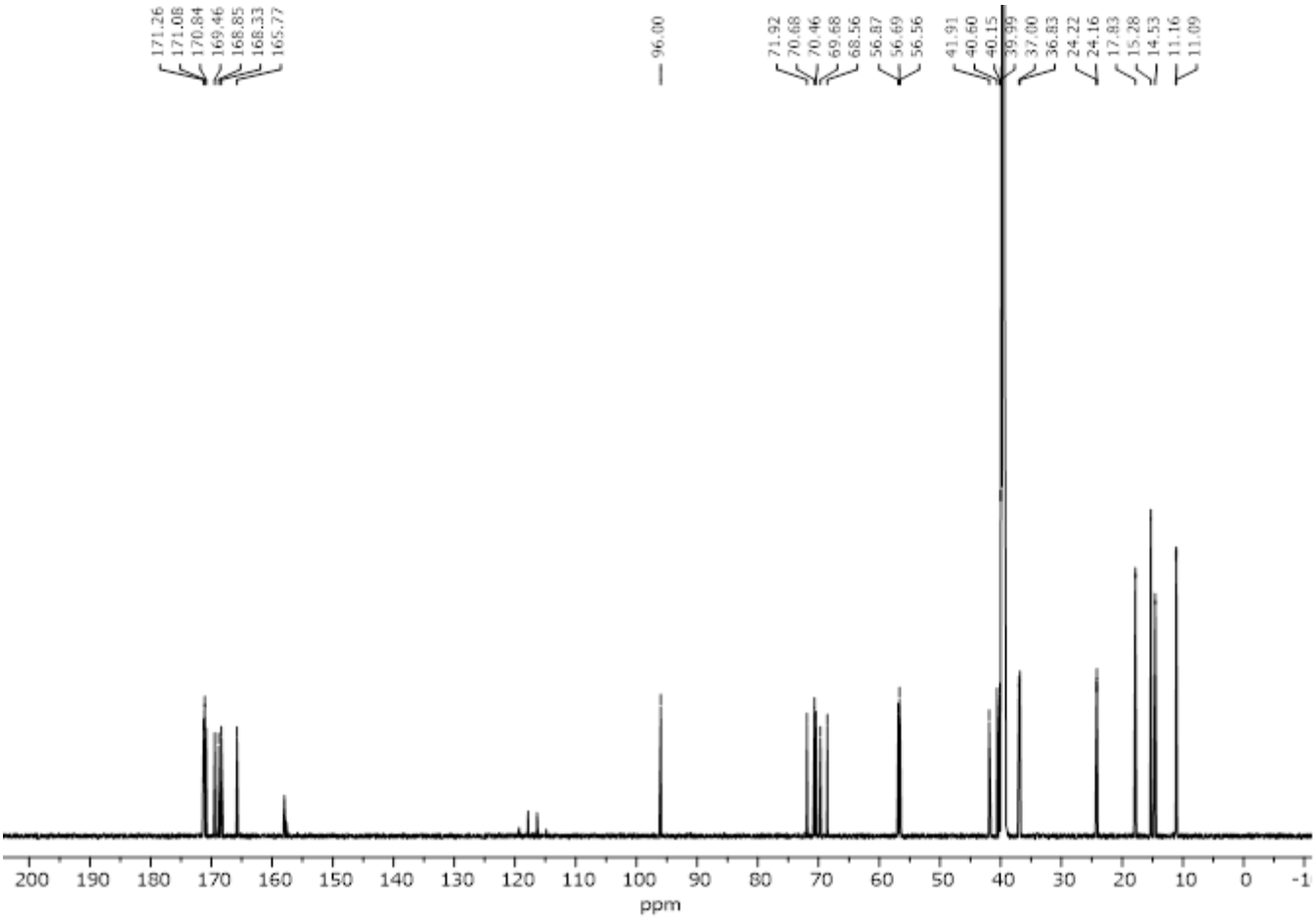


Analytical HPLC-trace of $Ser^{\beta\text{-}Rha}$-Peptide **12**

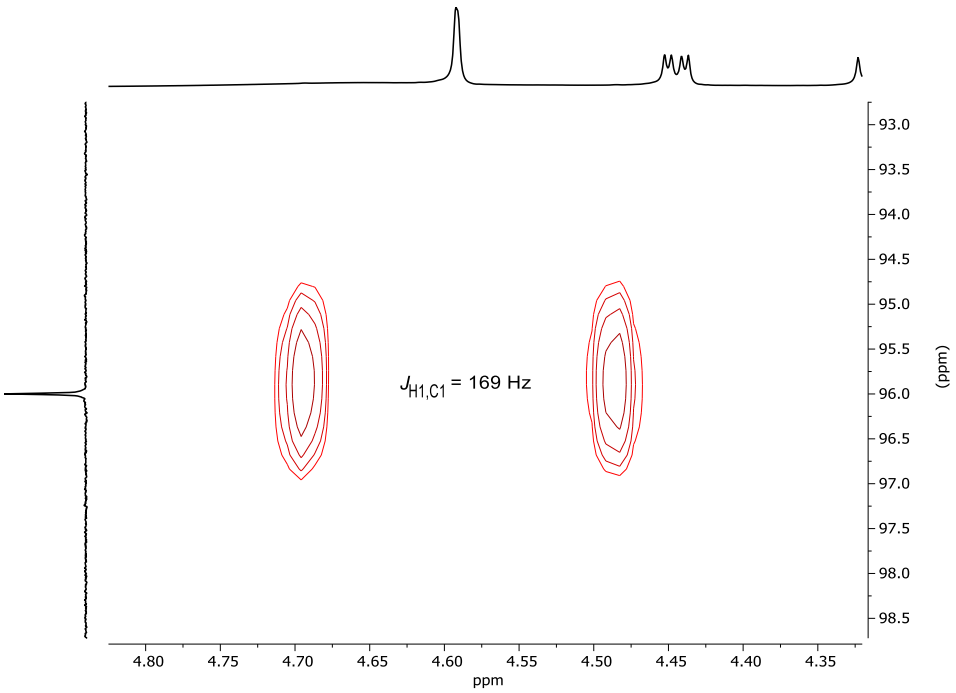
Thr$^{\alpha\text{-Rha}}$-Peptide **13**



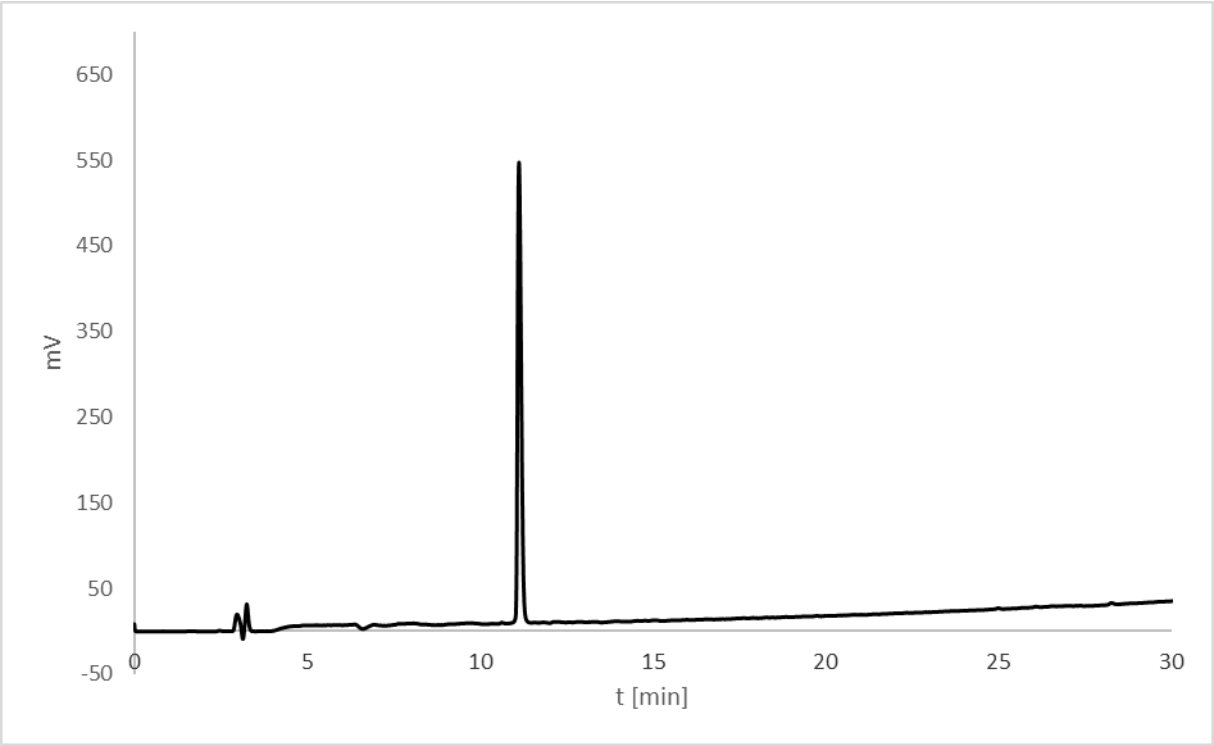
^{1}H-NMR Spectrum of Thr$^{\alpha\text{-Rha}}$-Peptide **13** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Thr$^{\alpha\text{-Rha}}$-Peptide **13** (200 MHz, [d6]-DMSO)

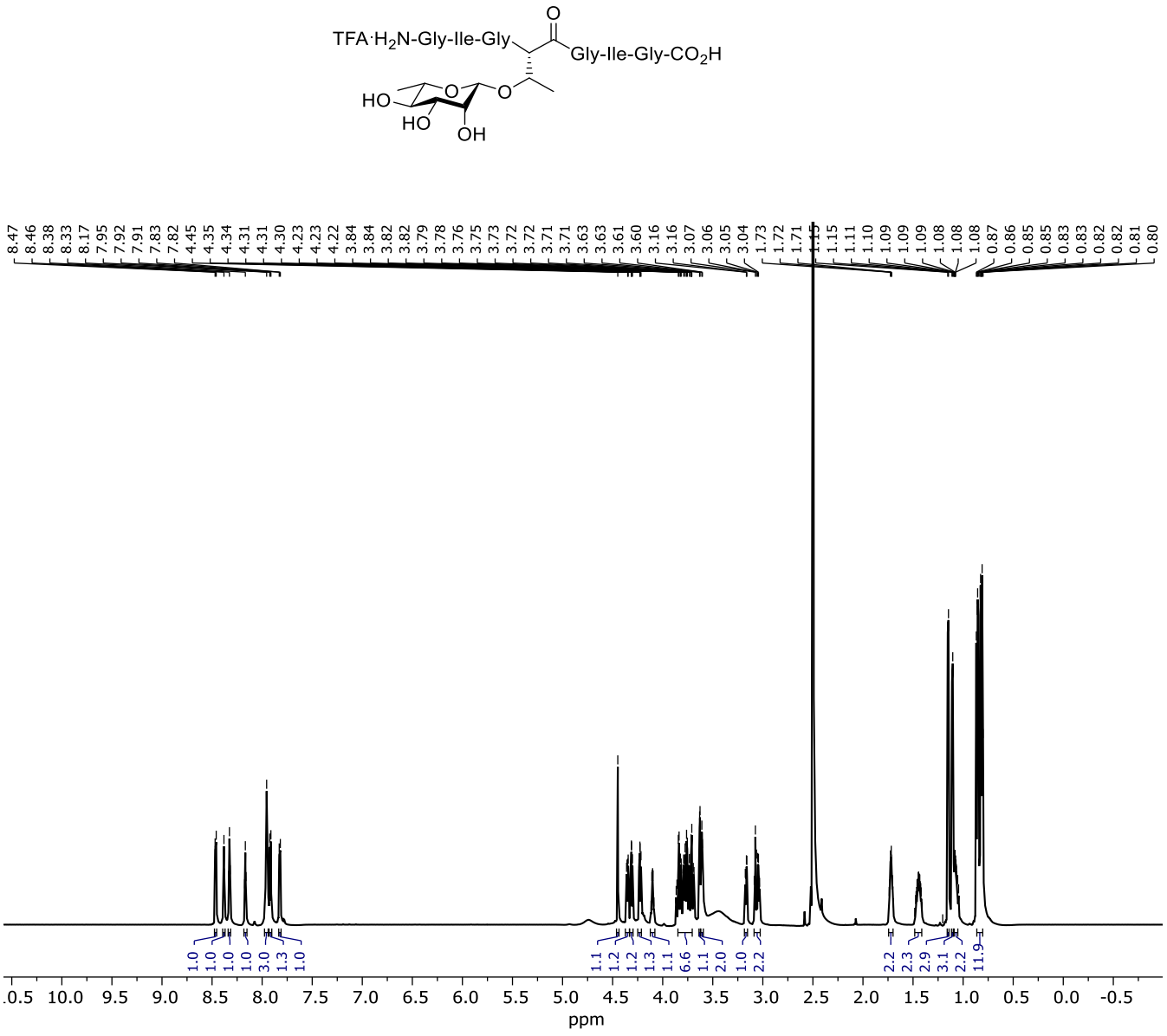


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **13**: H-1,C-1 coupling constant $J_{H1,C1}$

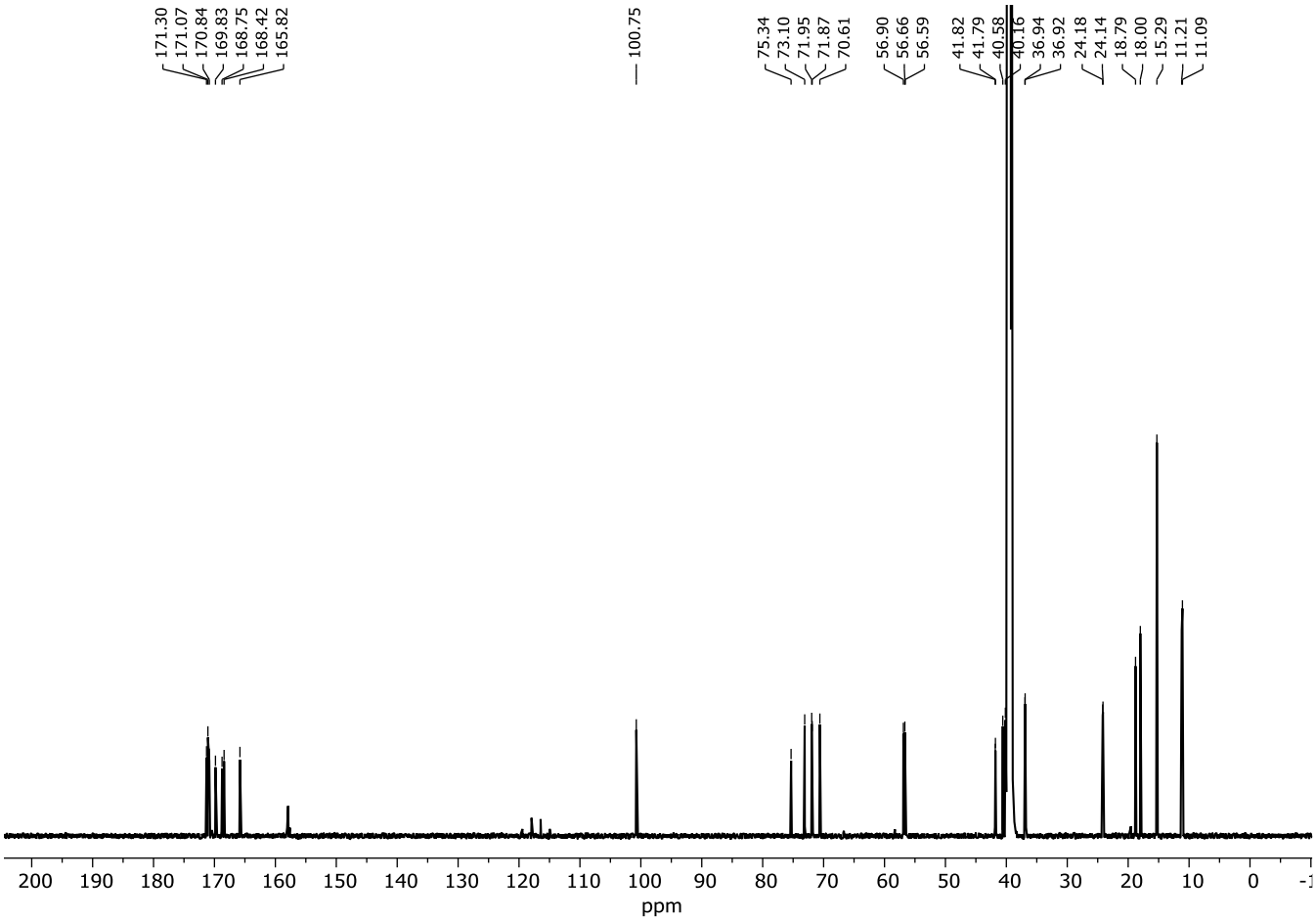


Analytical HPLC-trace of Thr$^{\alpha\text{-Rha}}$-Peptide **13**

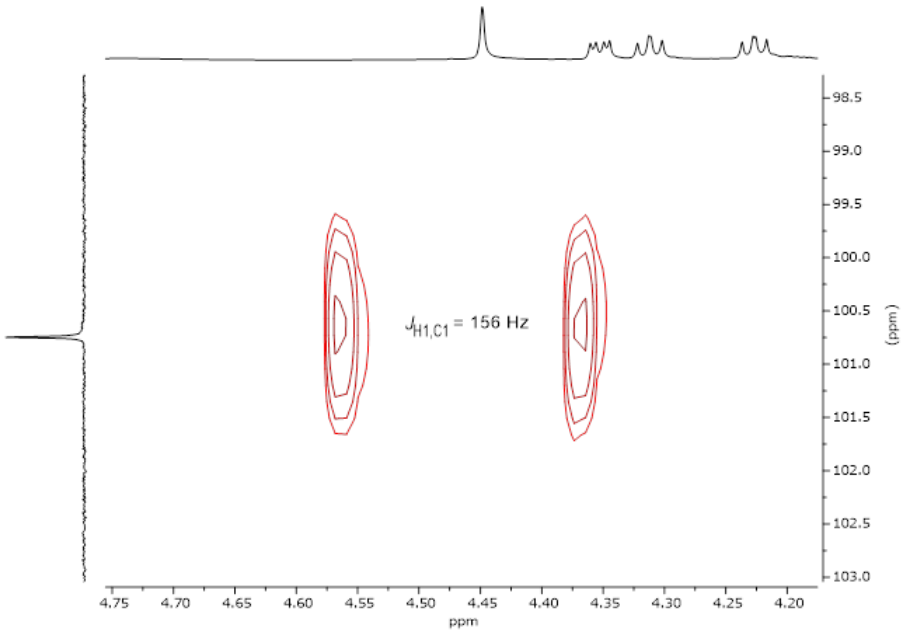
Thr$^{\beta\text{-Rha}}$-Peptide **14**



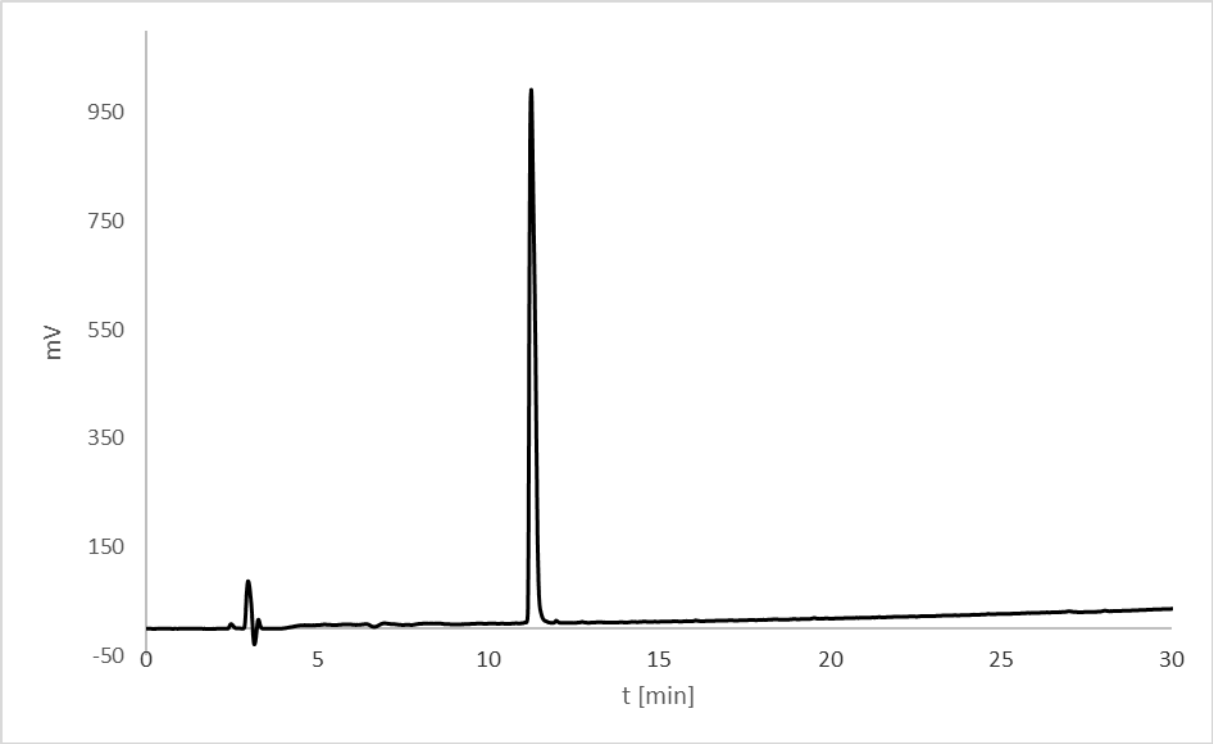
^{1}H-NMR Spectrum of Thr$^{\beta\text{-Rha}}$-Peptide **14** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Thr$^{\beta\text{-Rha}}$-Peptide **14** (200 MHz, [d6]-DMSO)

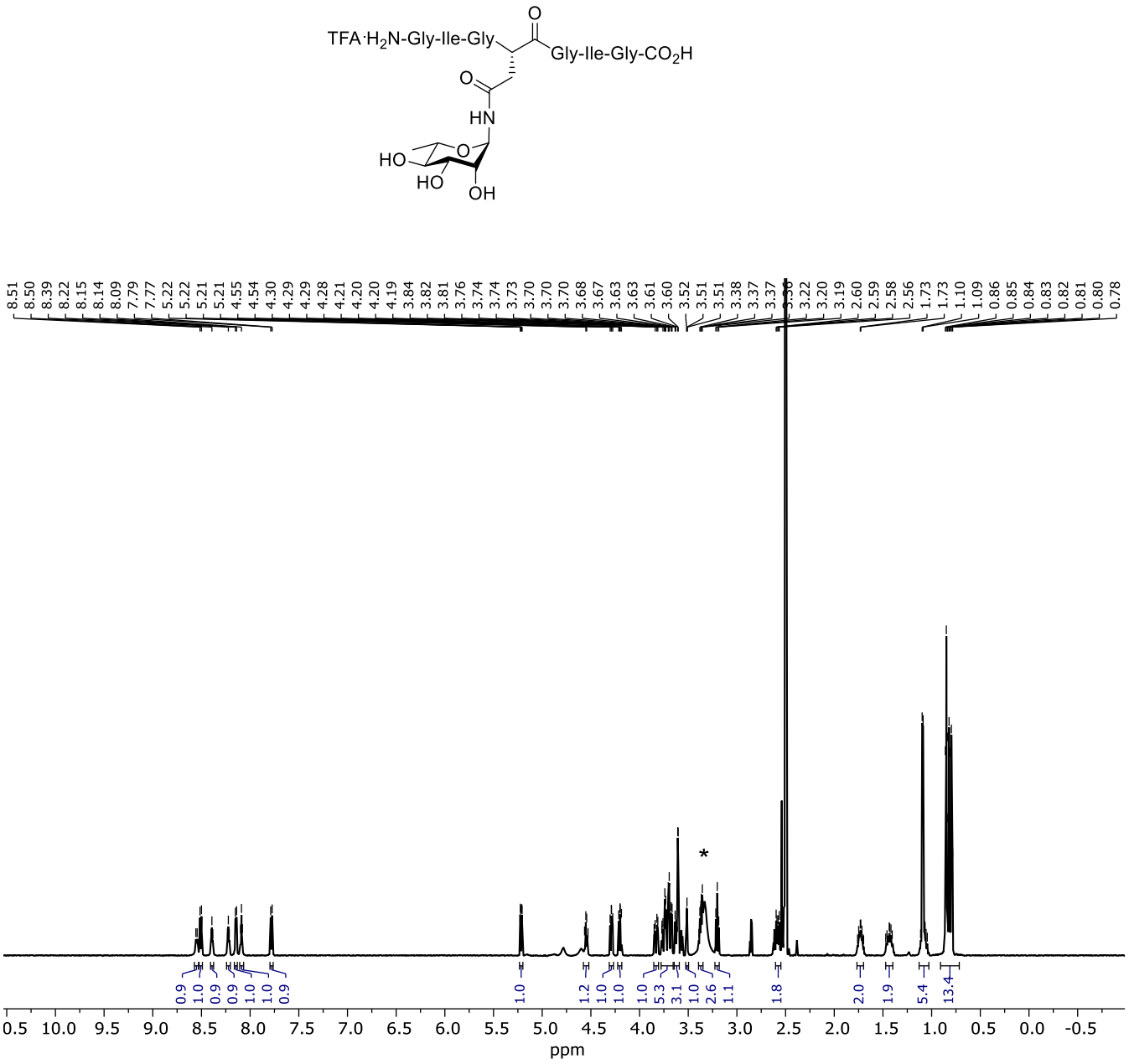


1H-13C-coupled HSQC-Spectrum of Glycopeptide **14**: H-1,C-1 coupling constant $J_{H1,C1}$

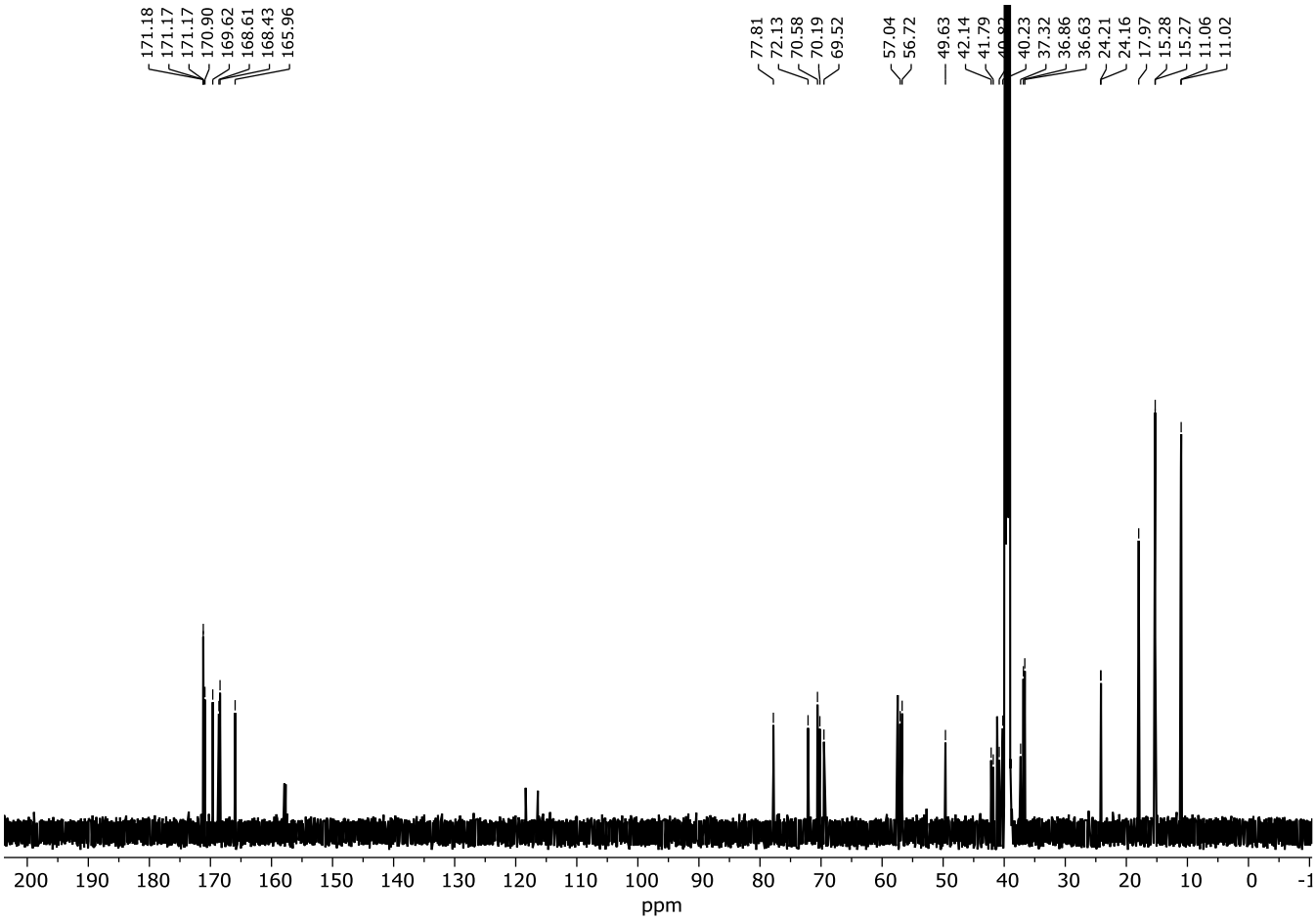


Analytical HPLC Trace of Thr$^{\beta\text{-Rha}}$-Peptide **14**

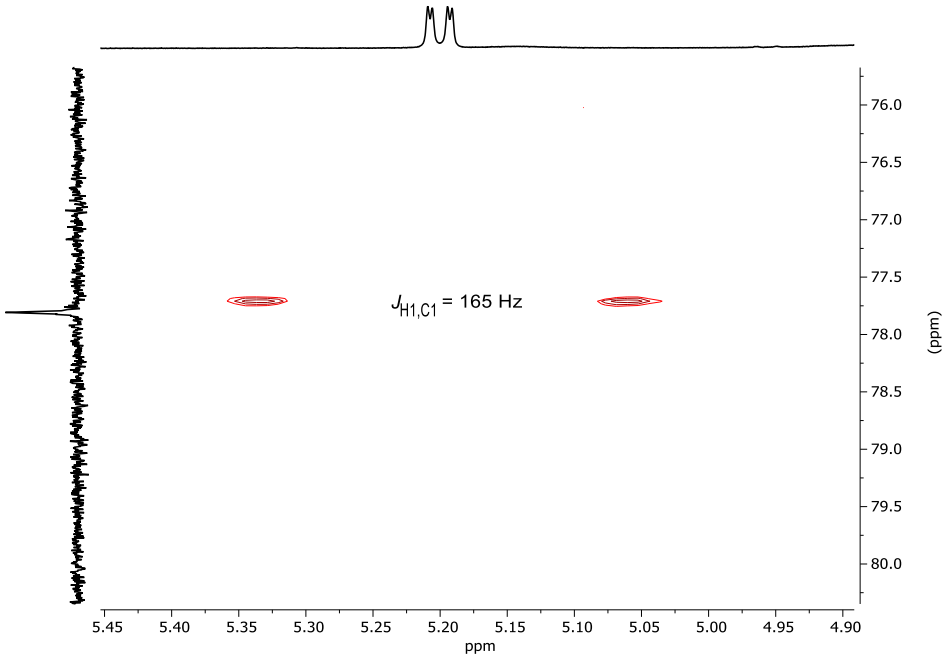
Asn$^{\alpha\text{-Rha}}$-Peptide **15**



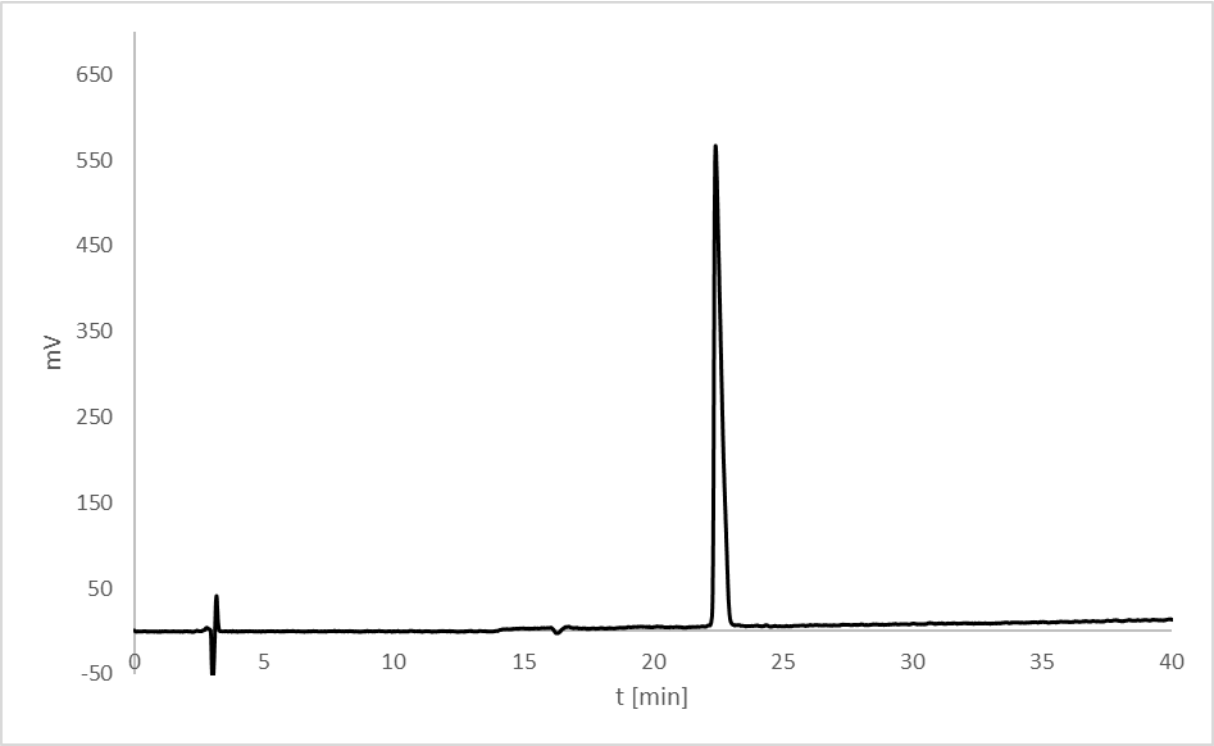
^{1}H-NMR Spectrum of Asn$^{\alpha\text{-Rha}}$-Peptide **15** (600 MHz, [d6]-DMSO); ***** Note: H-5 and Water peak.



^{13}C-NMR Spectrum of Asn$^{\alpha\text{-Rha}}$-Peptide **15** (150 MHz, [d6]-DMSO)

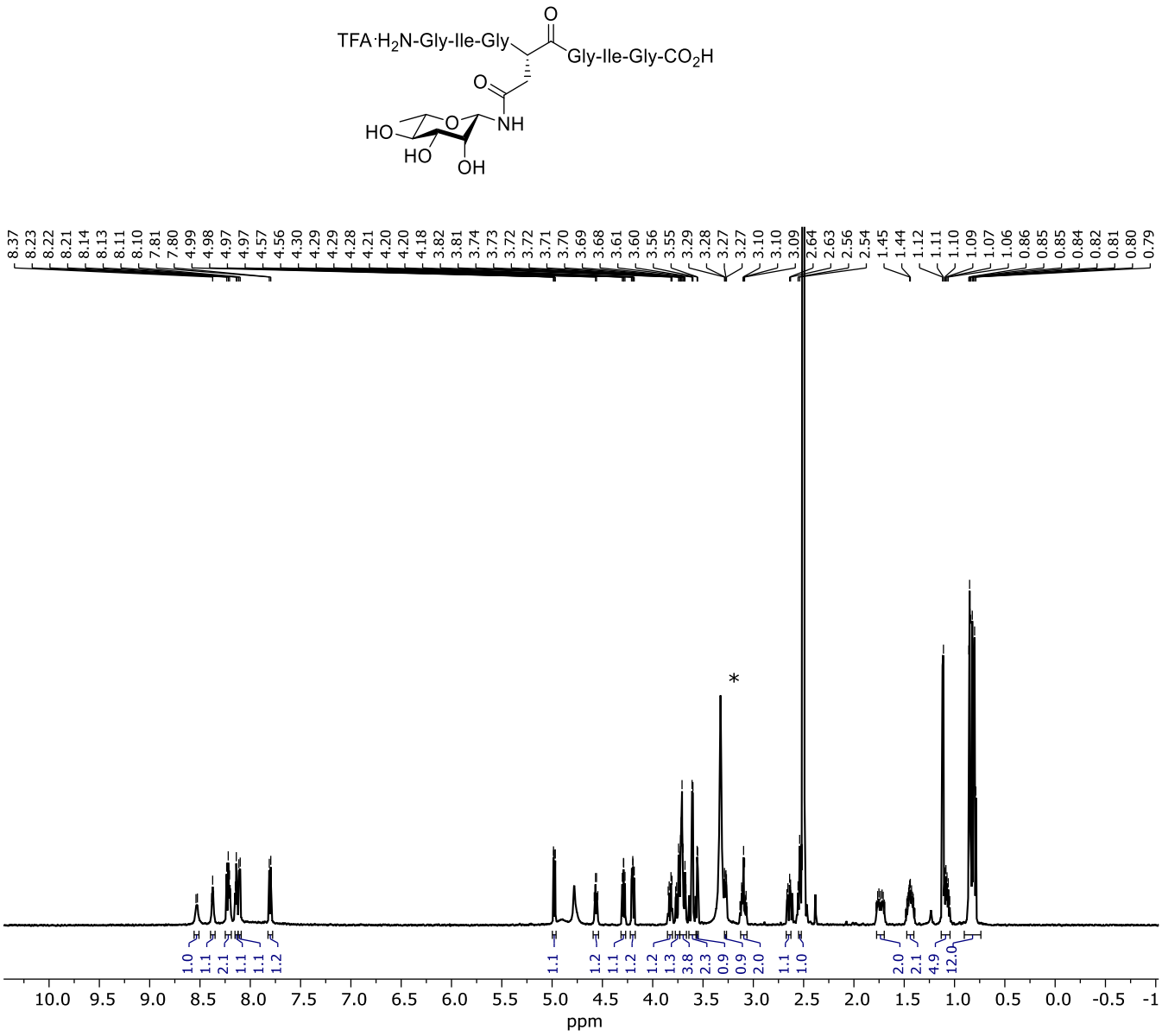


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **15**: H-1-C-1 coupling constant $J_{H1,C1}$

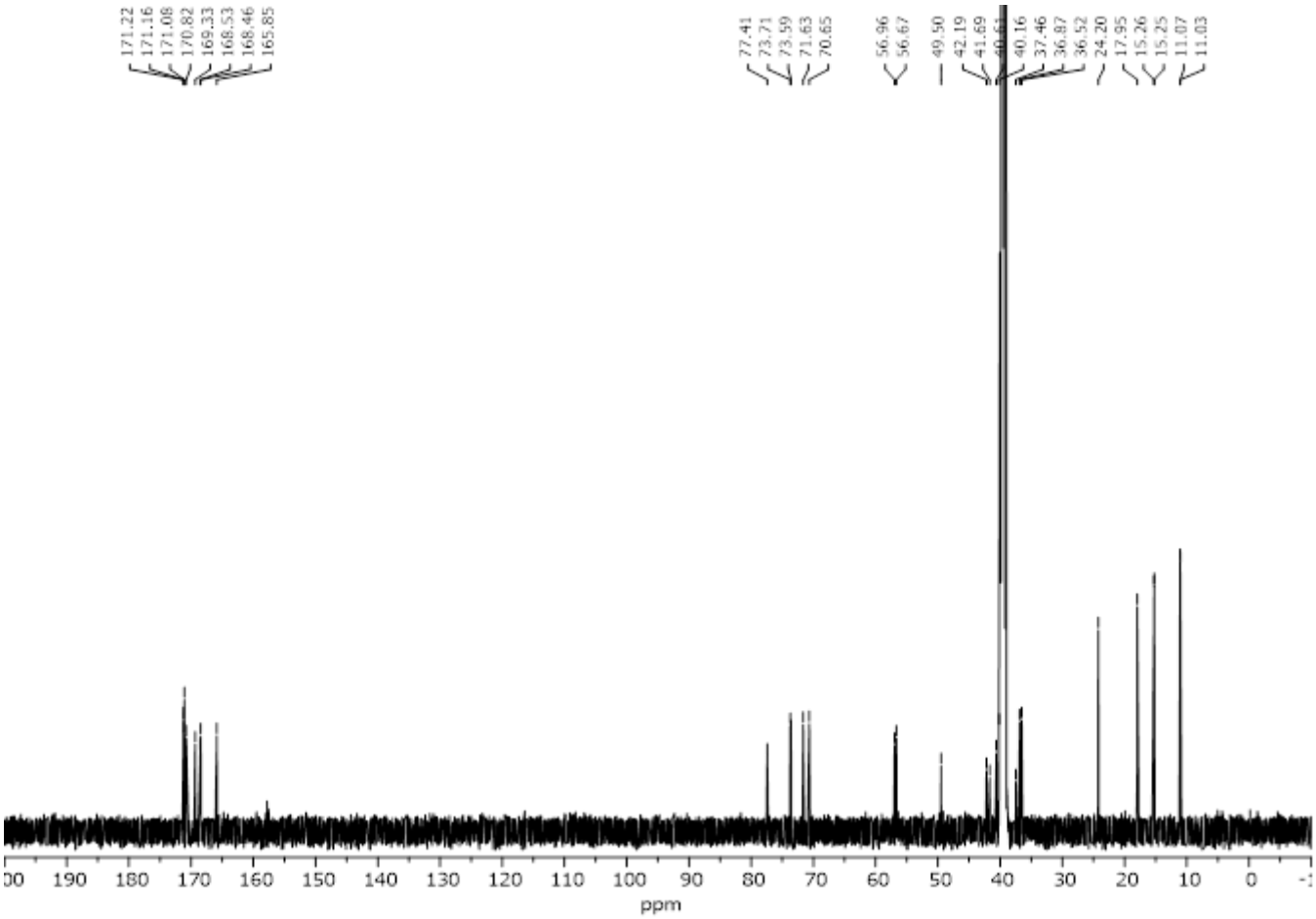


Analytical HPLC-trace of Asn$^{\alpha\text{-Rha}}$-Peptide **15**

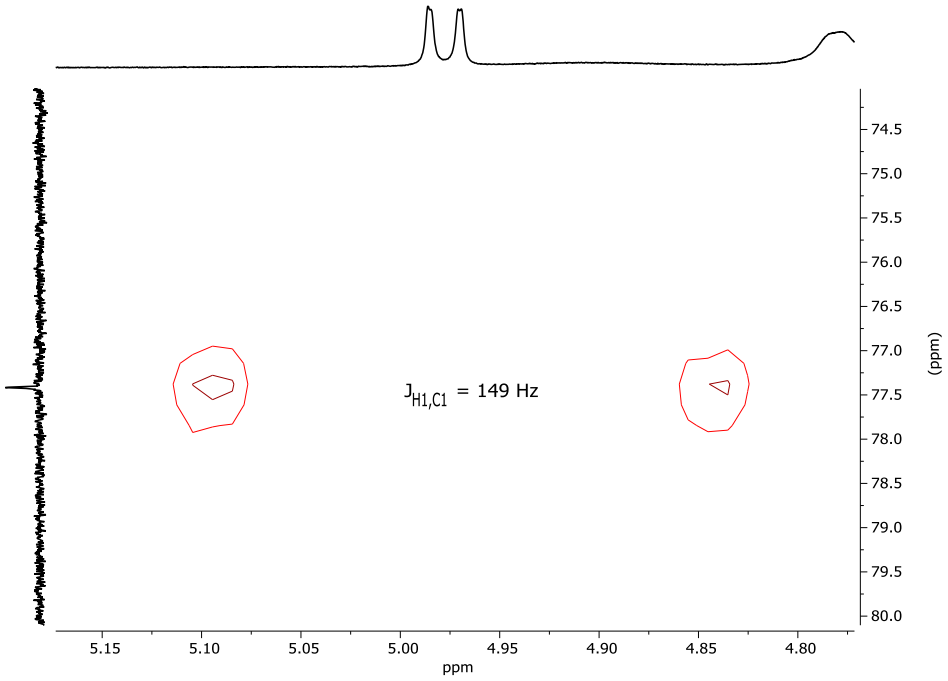
Asn$^{\beta\text{-Rha}}$-Peptide **16**



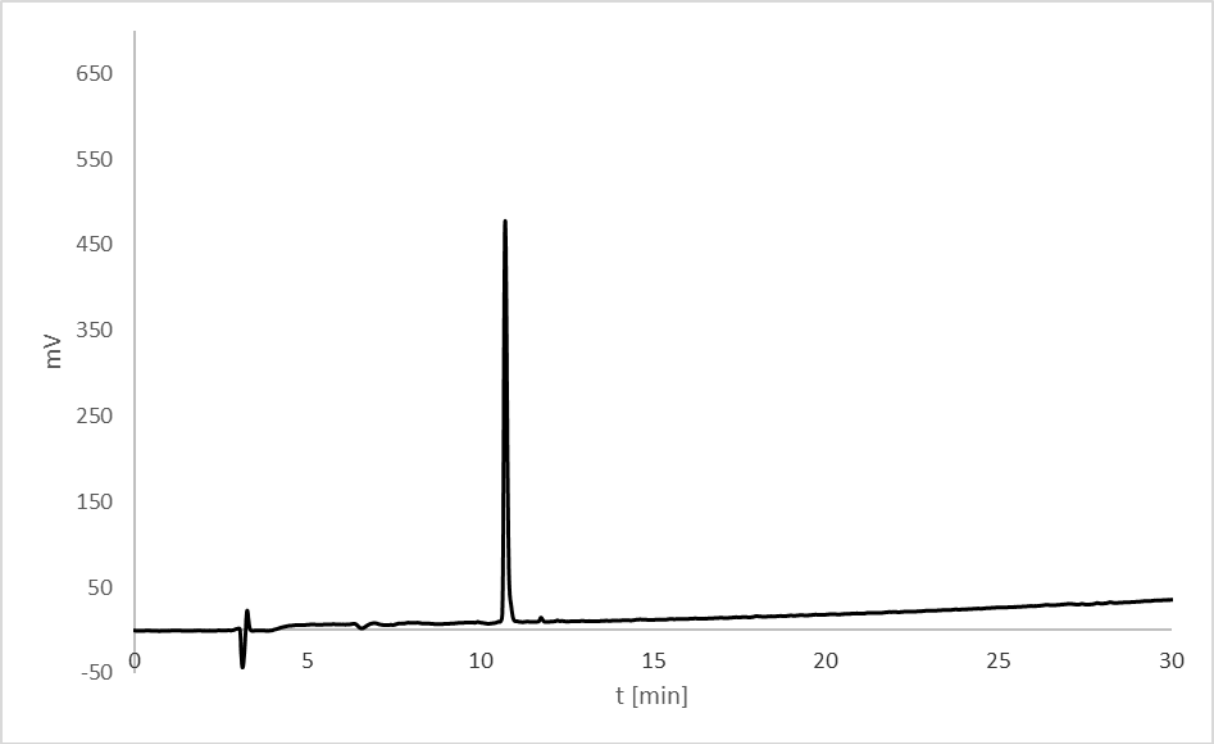
^{1}H-NMR Spectrum of Asn$^{\beta\text{-Rha}}$-Peptide **16** (600 MHz, [d6]-DMSO); * H_2O



^{13}C-NMR Spectrum of Asn$^{\beta\text{-Rha}}$-Peptide **16** (150 MHz, [d6]-DMSO)

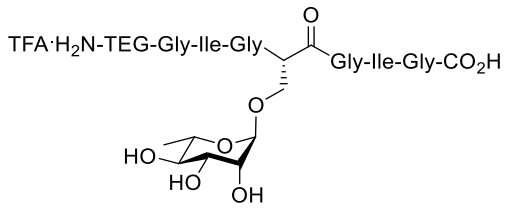


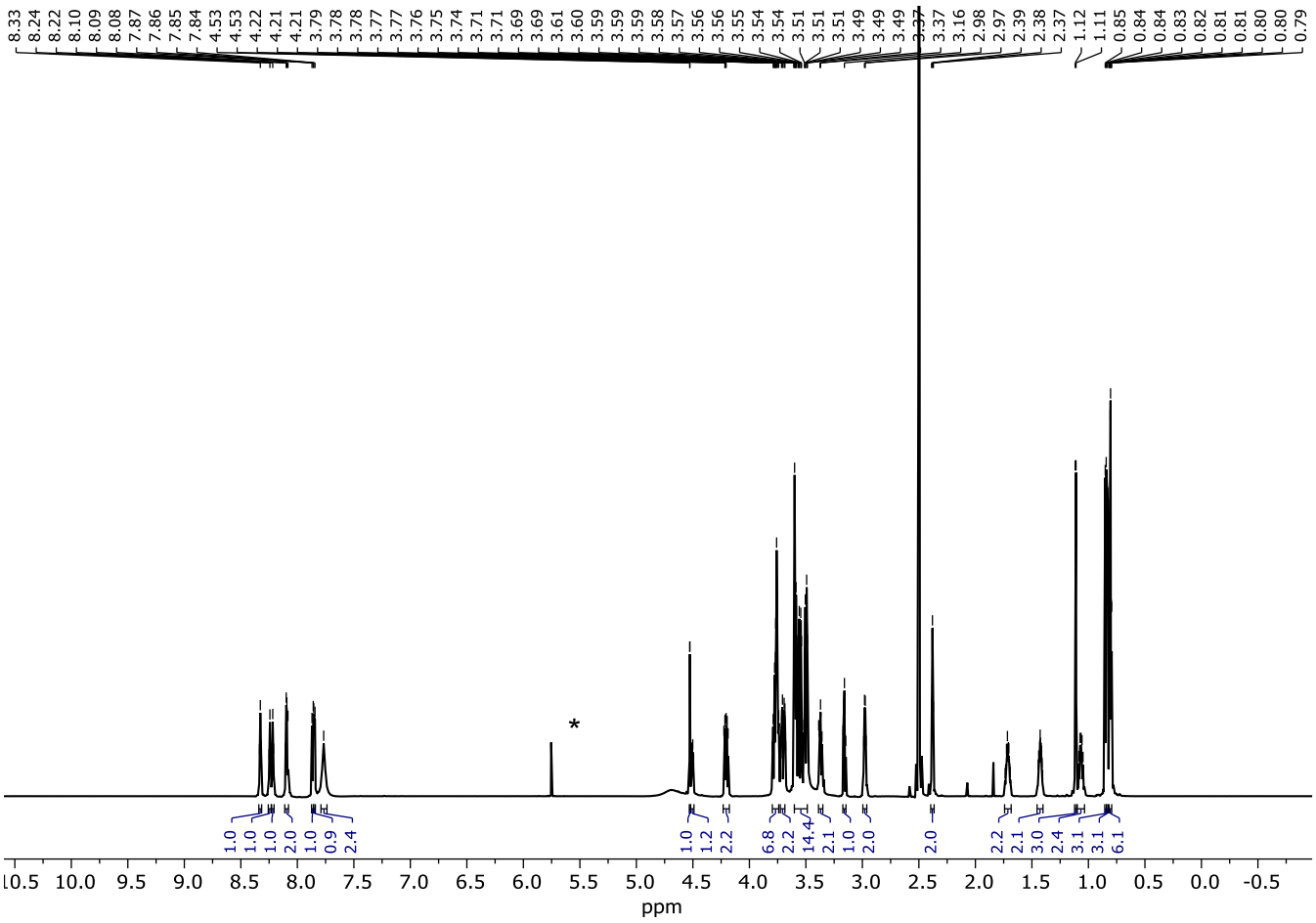
^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **16**: H-1-C-1 coupling constant $J_{H1,C1}$



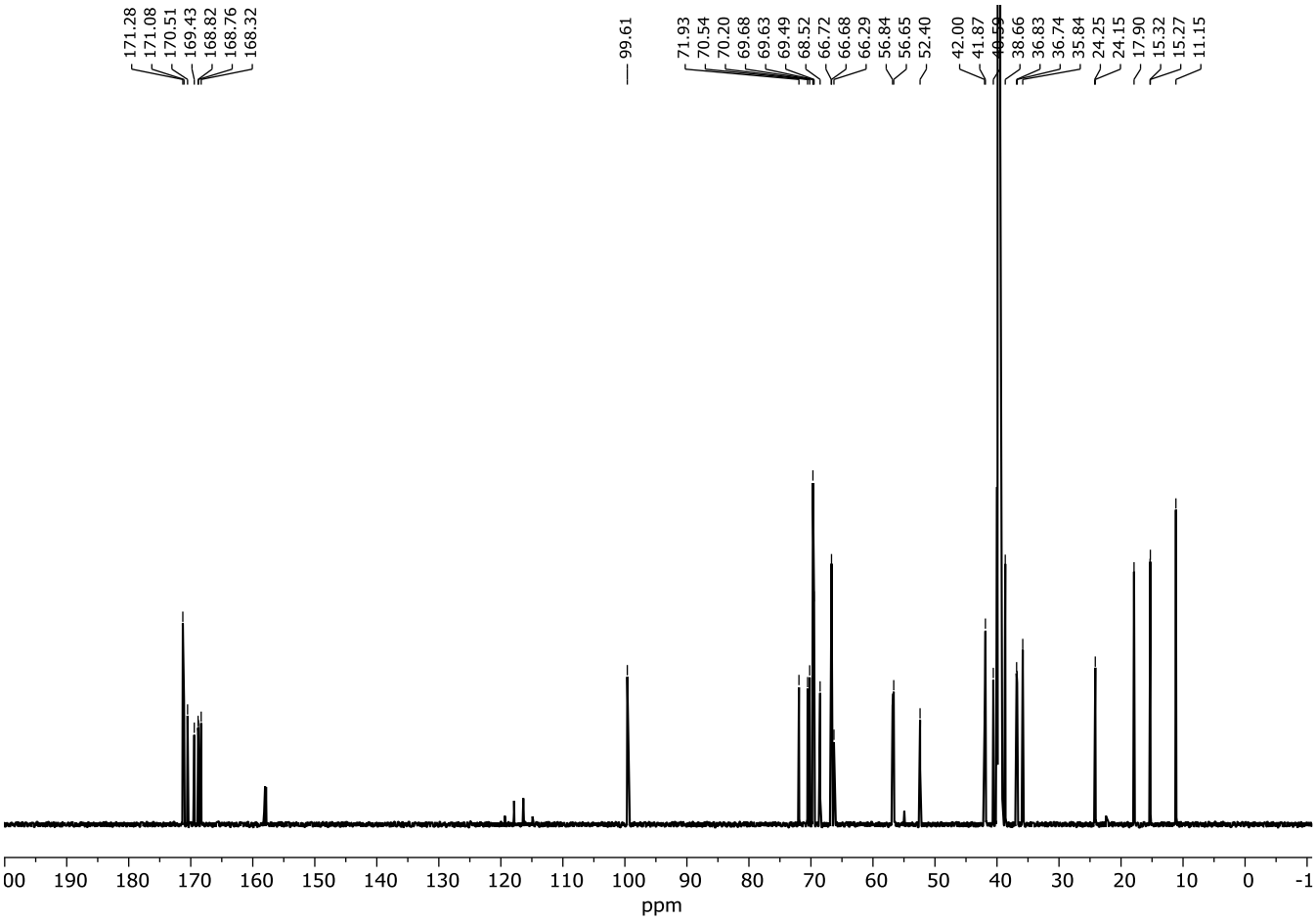
Analytical HPLC-trace of Asn$^{\beta\text{-Rha}}$-Peptide **16**

Ser$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S11**

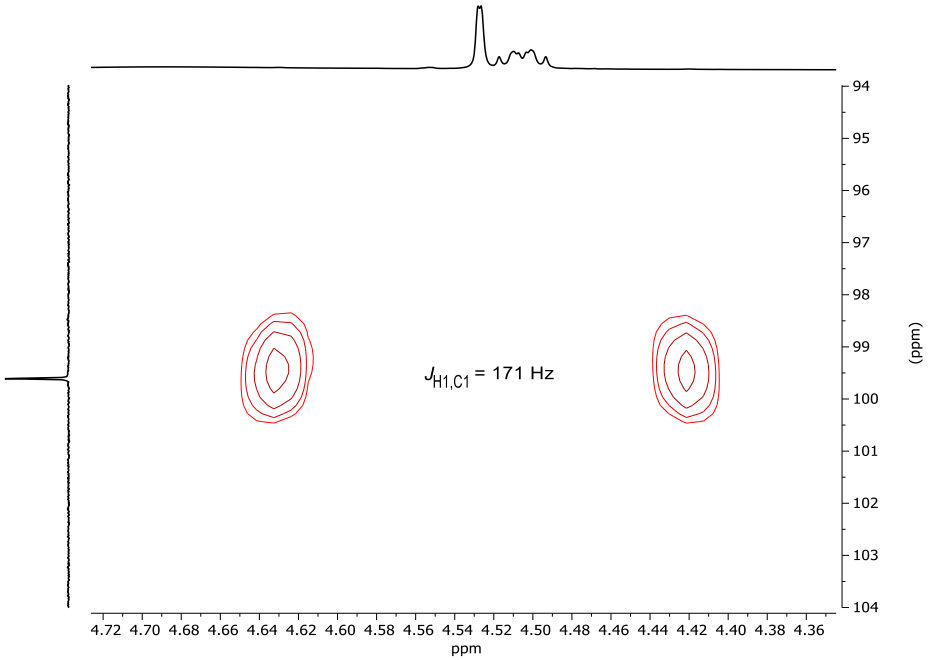




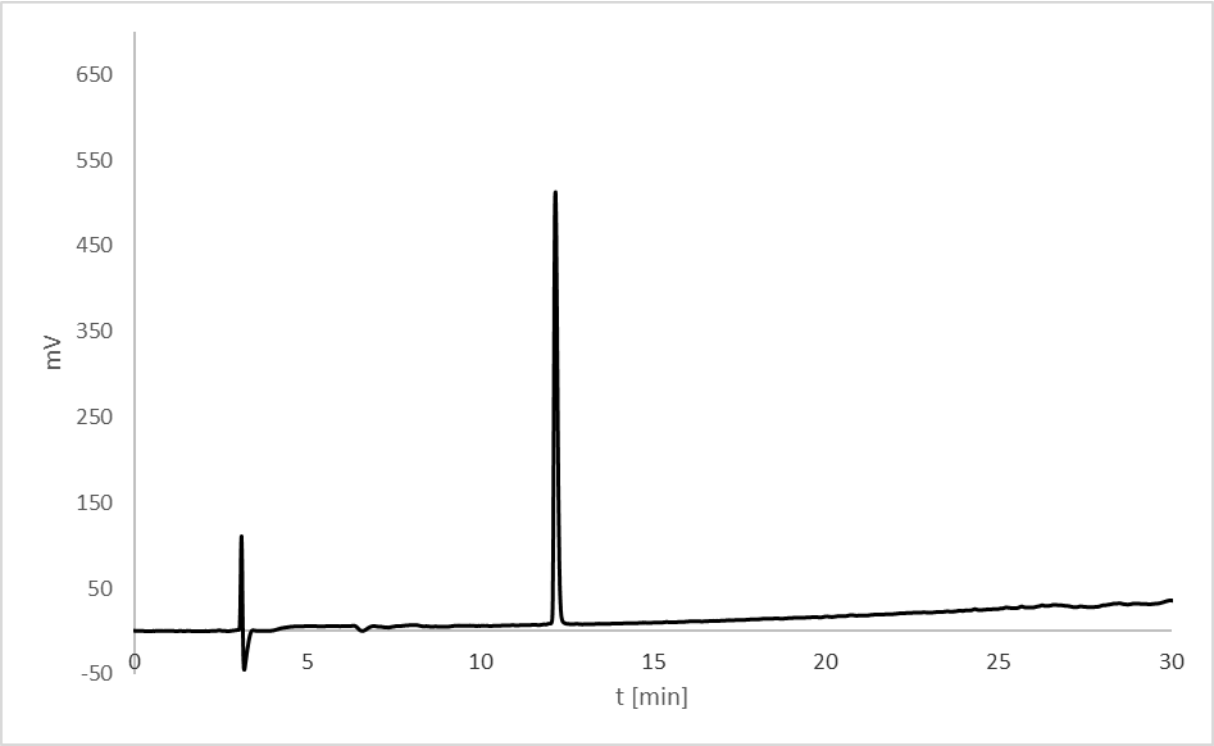
^{1}H-NMR Spectrum of Ser$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S11** (800 MHz, [d6]-DMSO); * CH_2Cl_2 impurity



^{13}C-NMR Spectrum of Ser$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S11** (200 MHz, [d6]-DMSO)

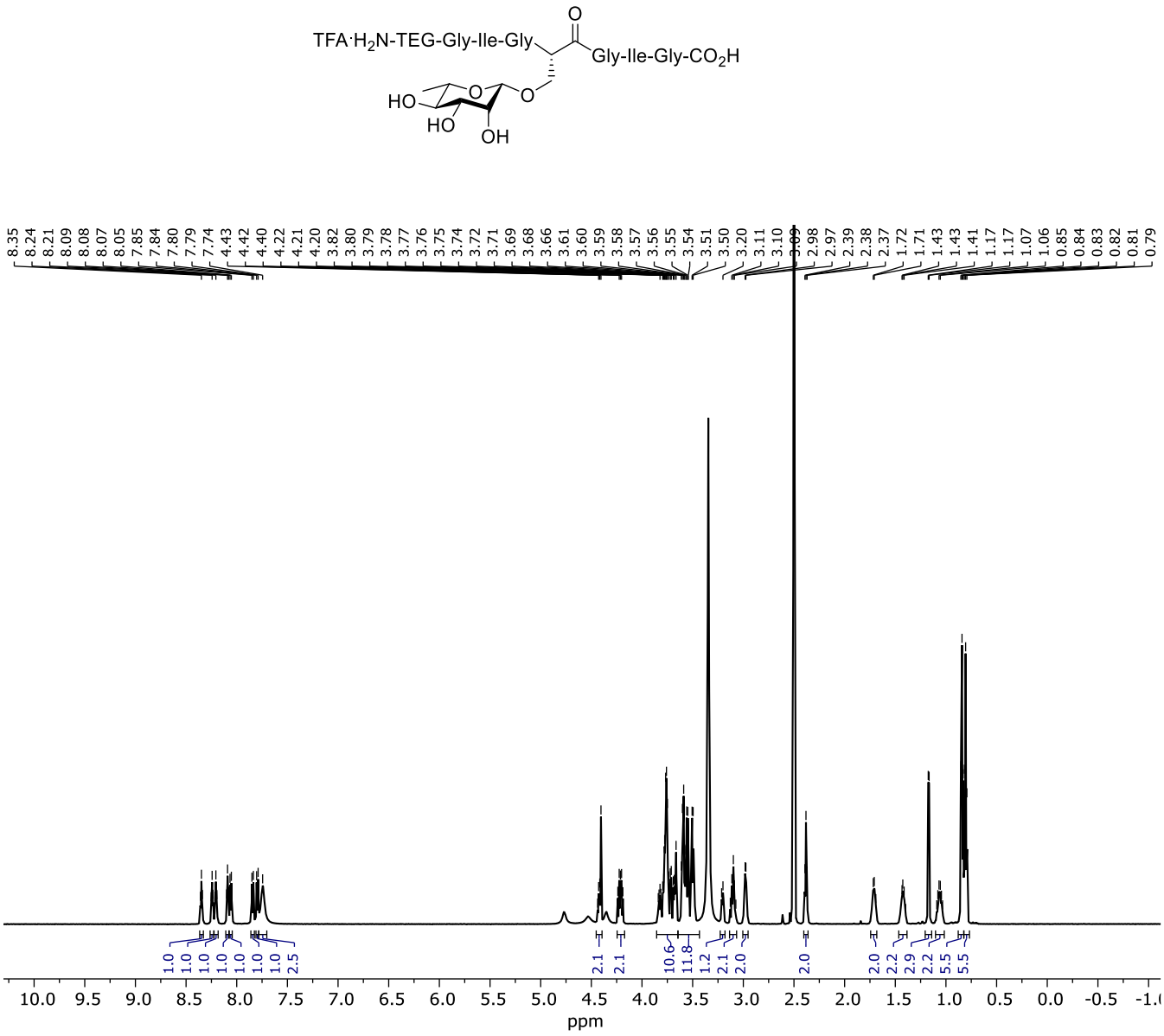


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **S11**: H-1,C-1 coupling constant $J_{H1,C1}$

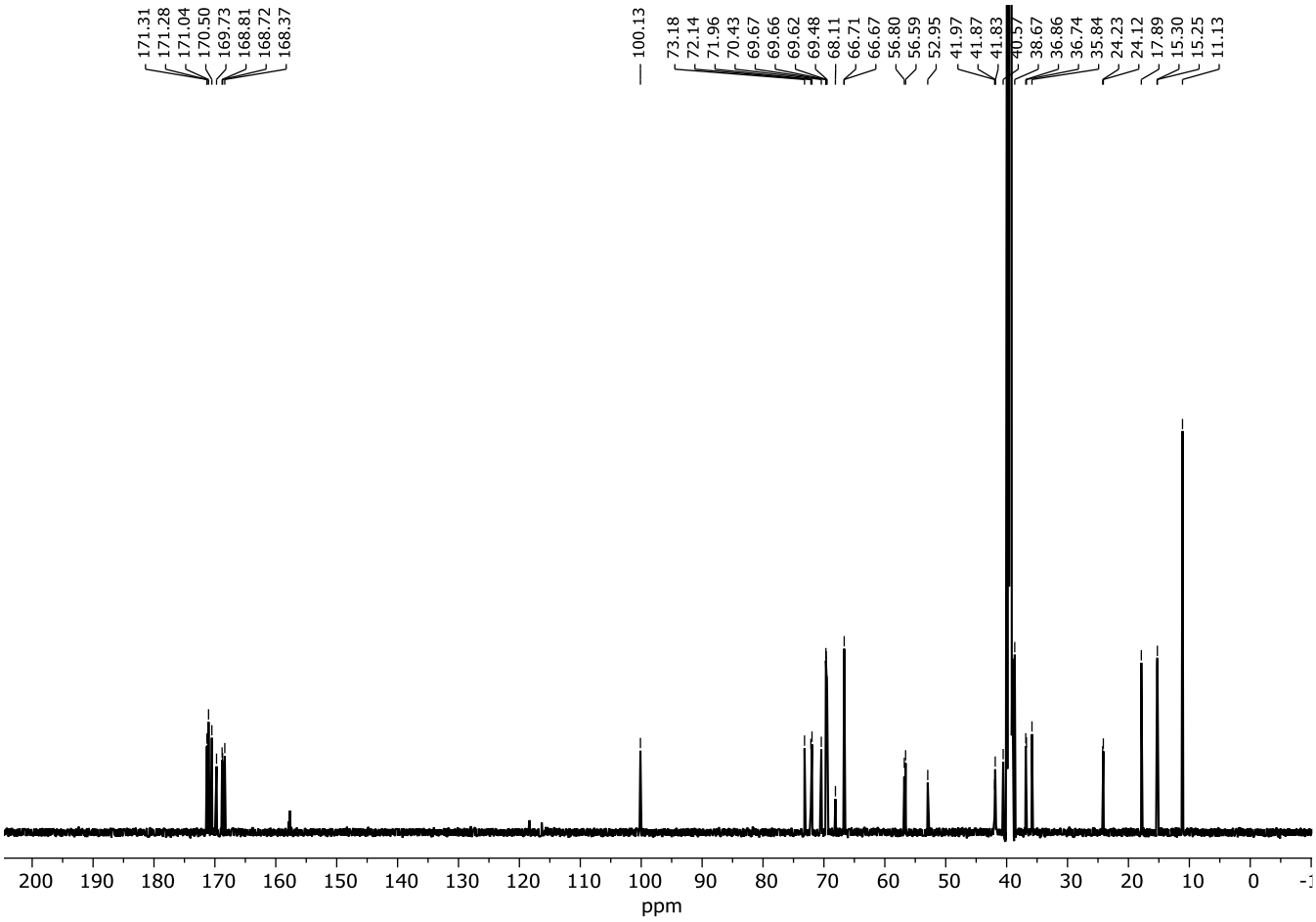


Analytical HPLC-trace of Ser$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S11**

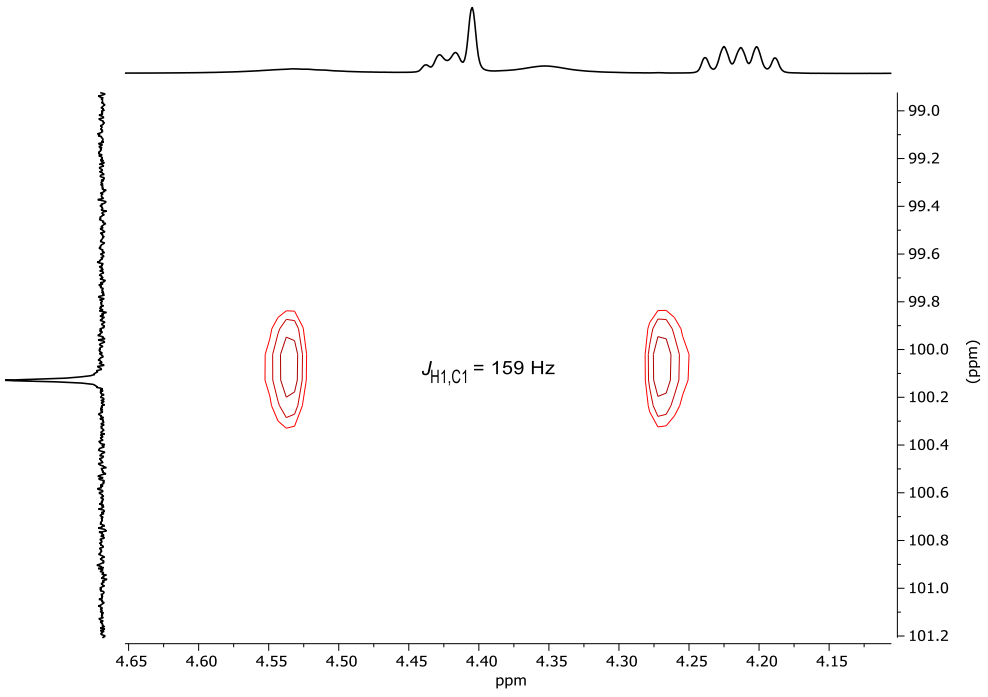
Ser$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S12**



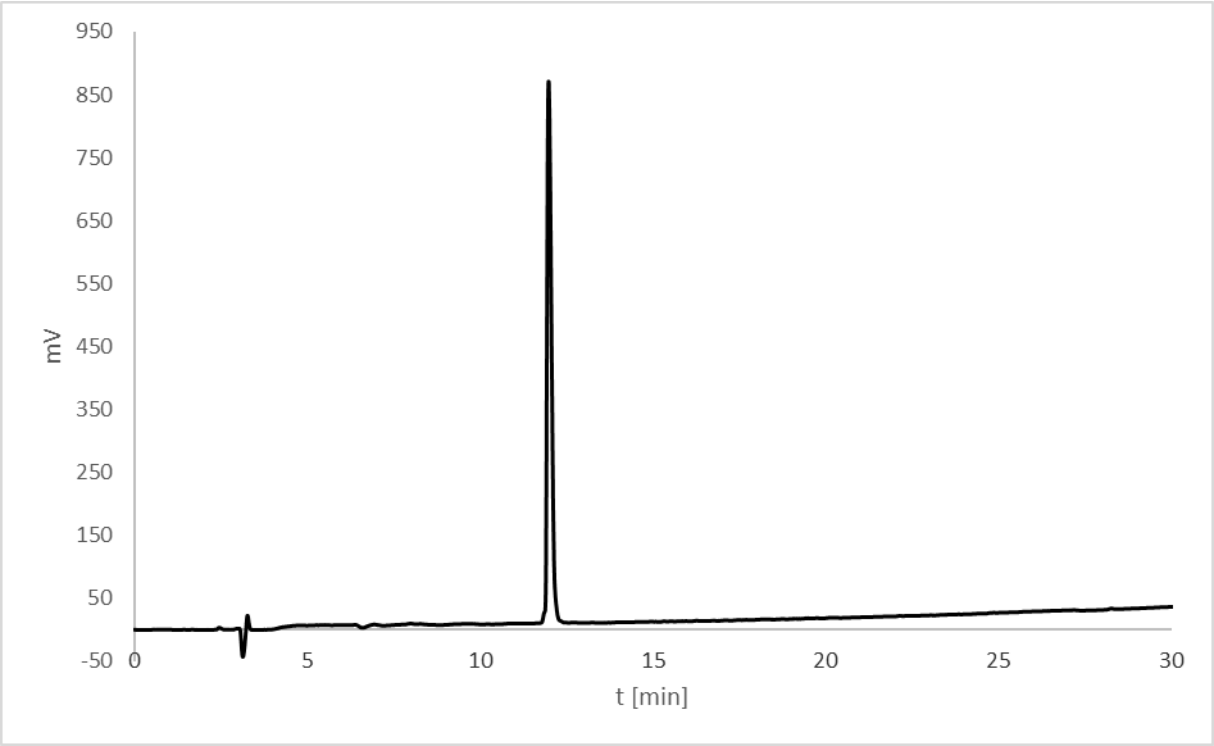
^{1}H-NMR Spectrum of Ser$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S12** (600 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum Ser$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S12** (150 MHz, [d6]-DMSO)

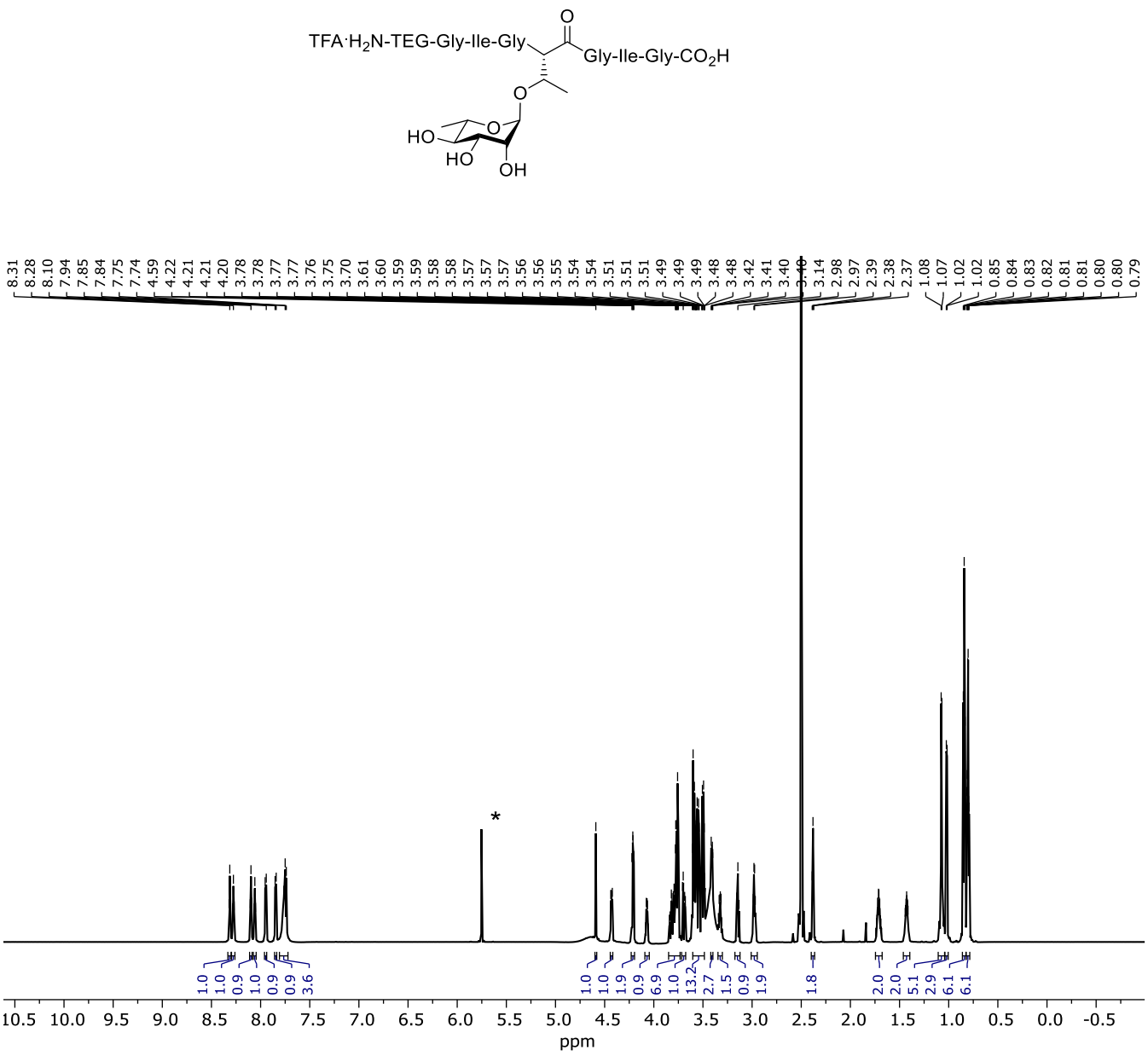


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **S12**: H-1,C-1 coupling constant $J_{H1,C1}$



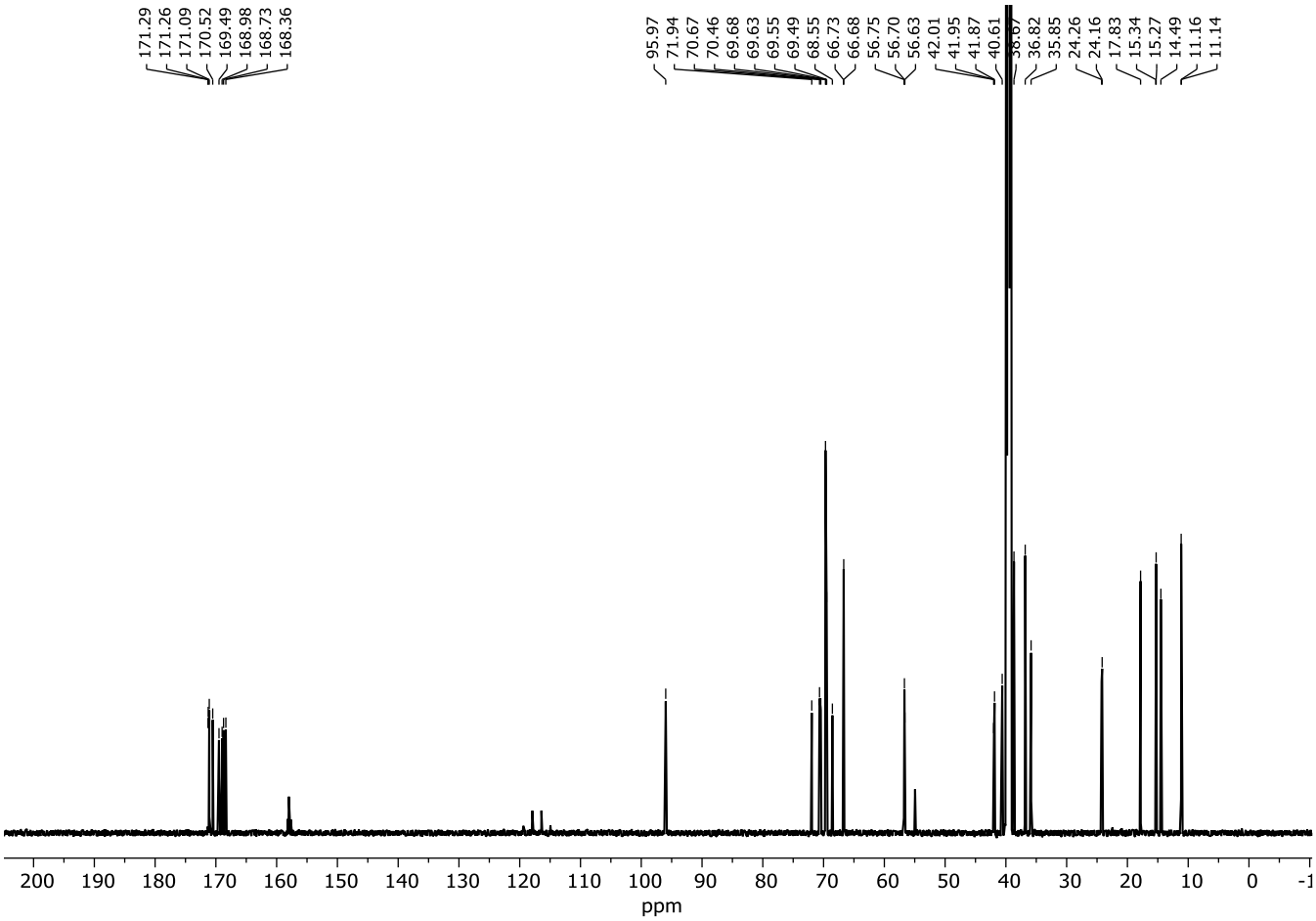
Analytical HPLC-trace of $Ser^{\beta\text{-}Rha}$-Peptide with TEG-Spacer **S12**

Thr$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer (**S13)**

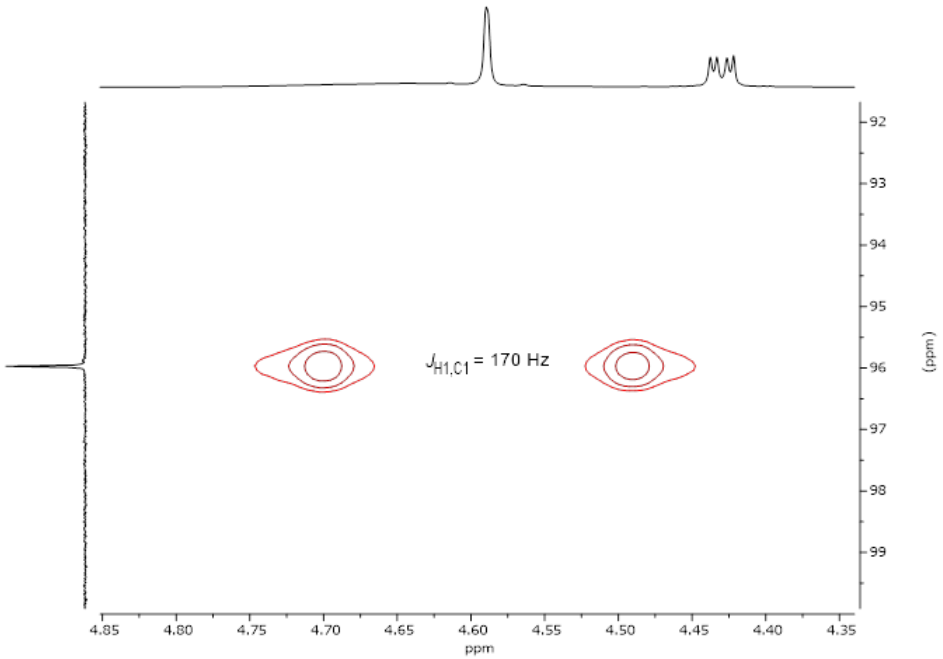


^{1}H-NMR Spectrum of Thr$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S13** (800 MHz, [d6]-DMSO);

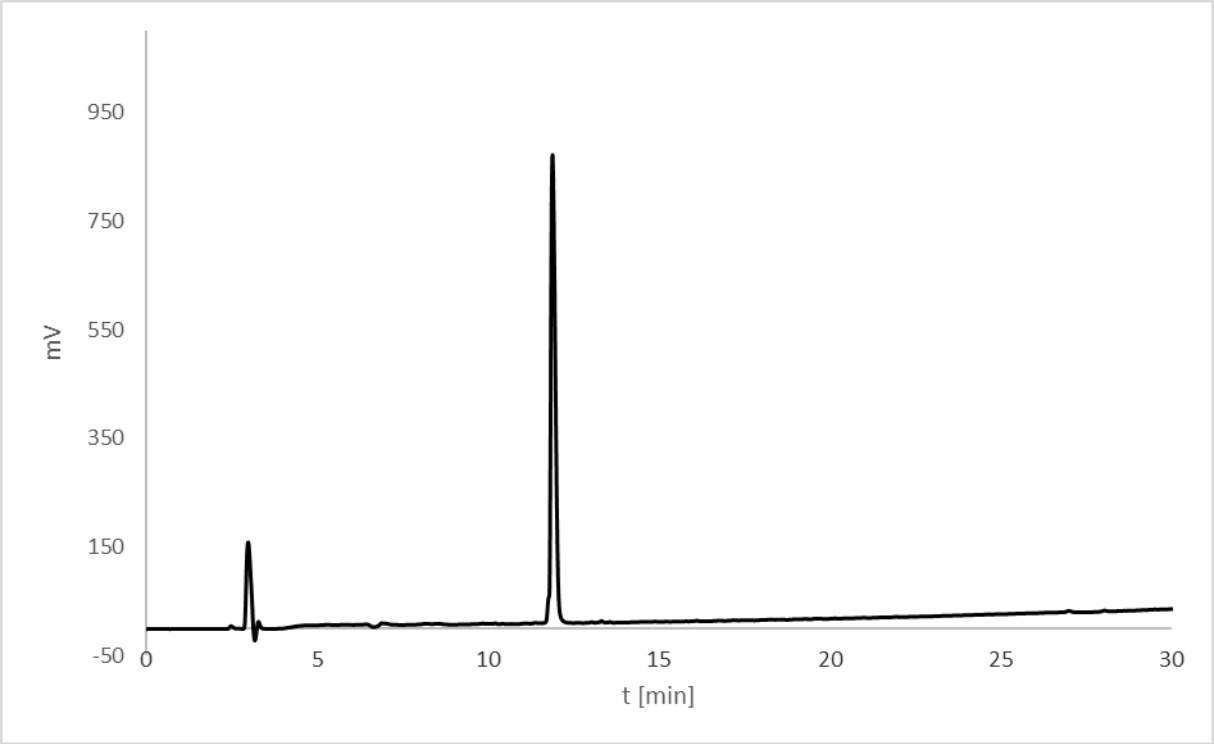
Note: *CH_2Cl_2



^{13}C-NMR Spectrum of Thr$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S13** (200 MHz, [d6]-DMSO)

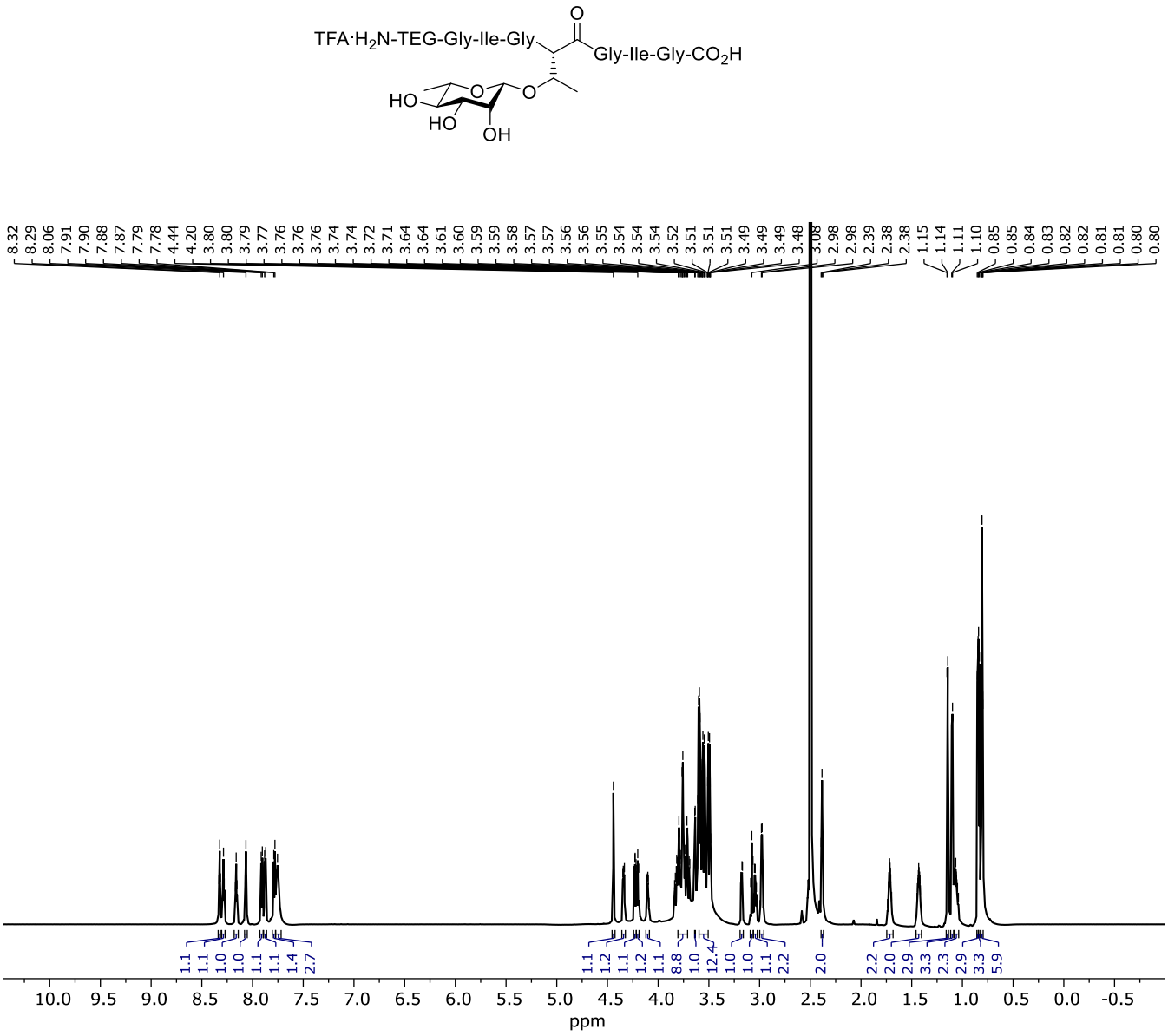


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **S13**: H-1,C-1 coupling constant $J_{H1,C1}$

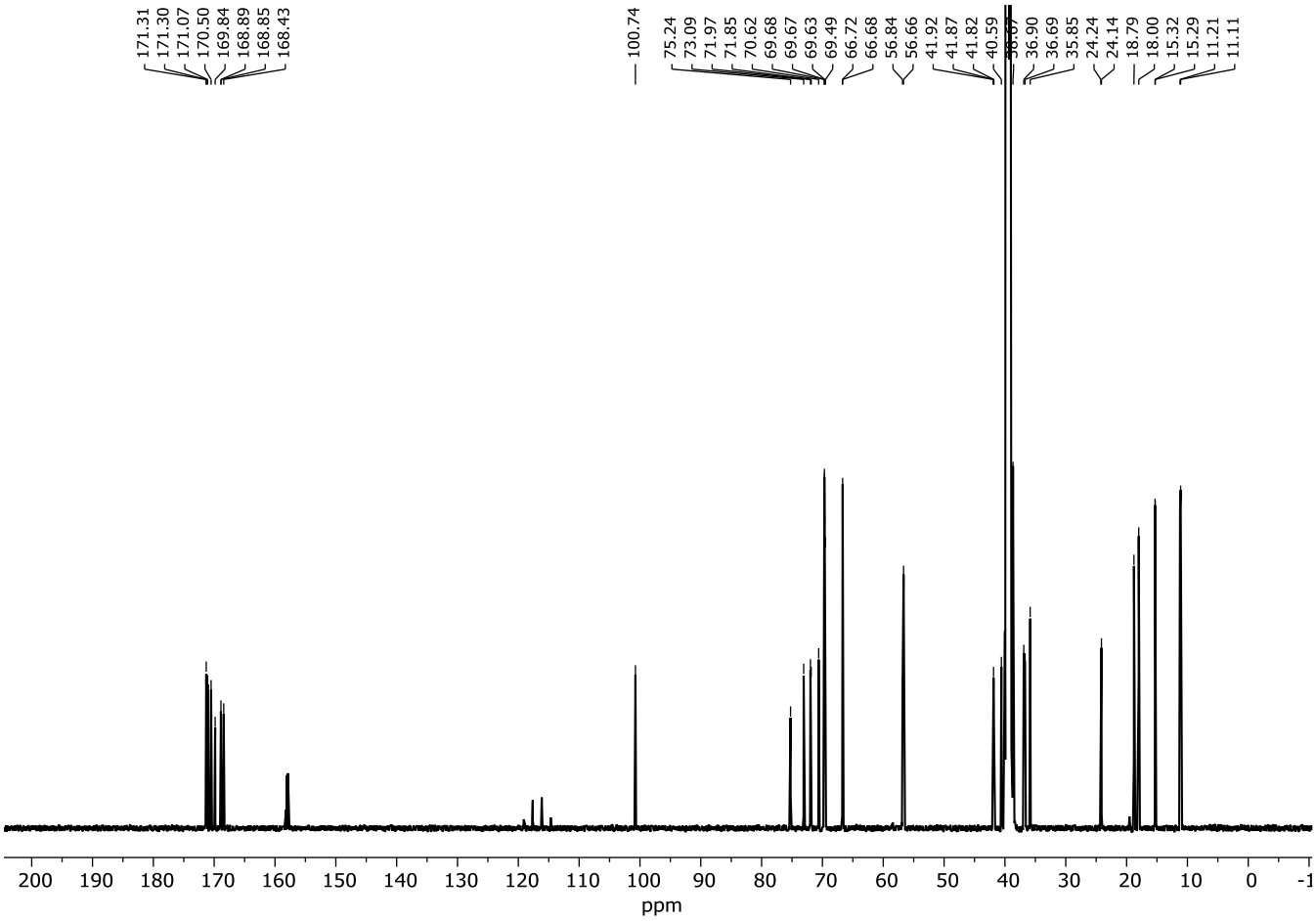


Analytical HPLC trace of Thr$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S13**

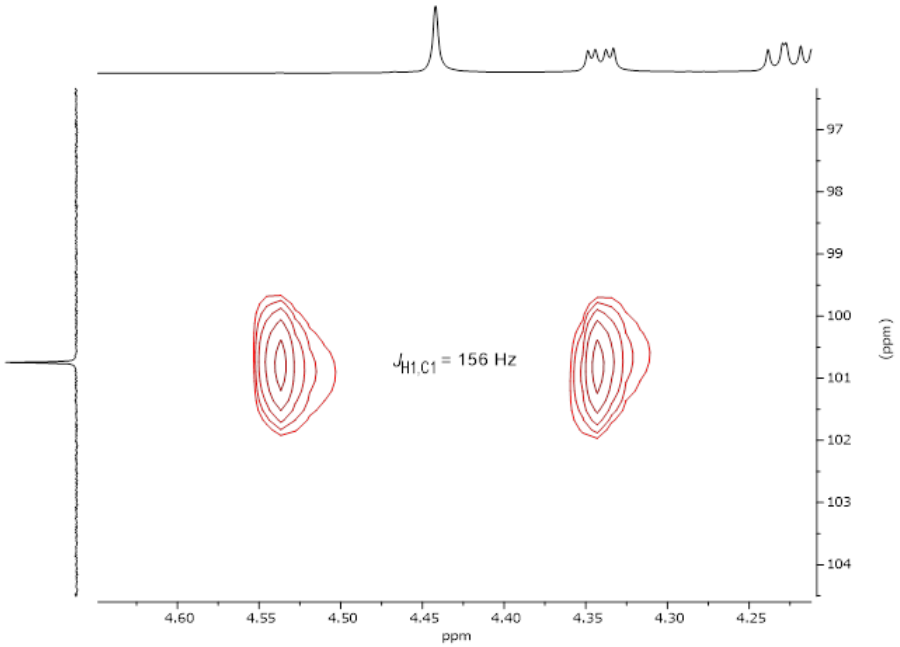
Thr$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer (**S14)**



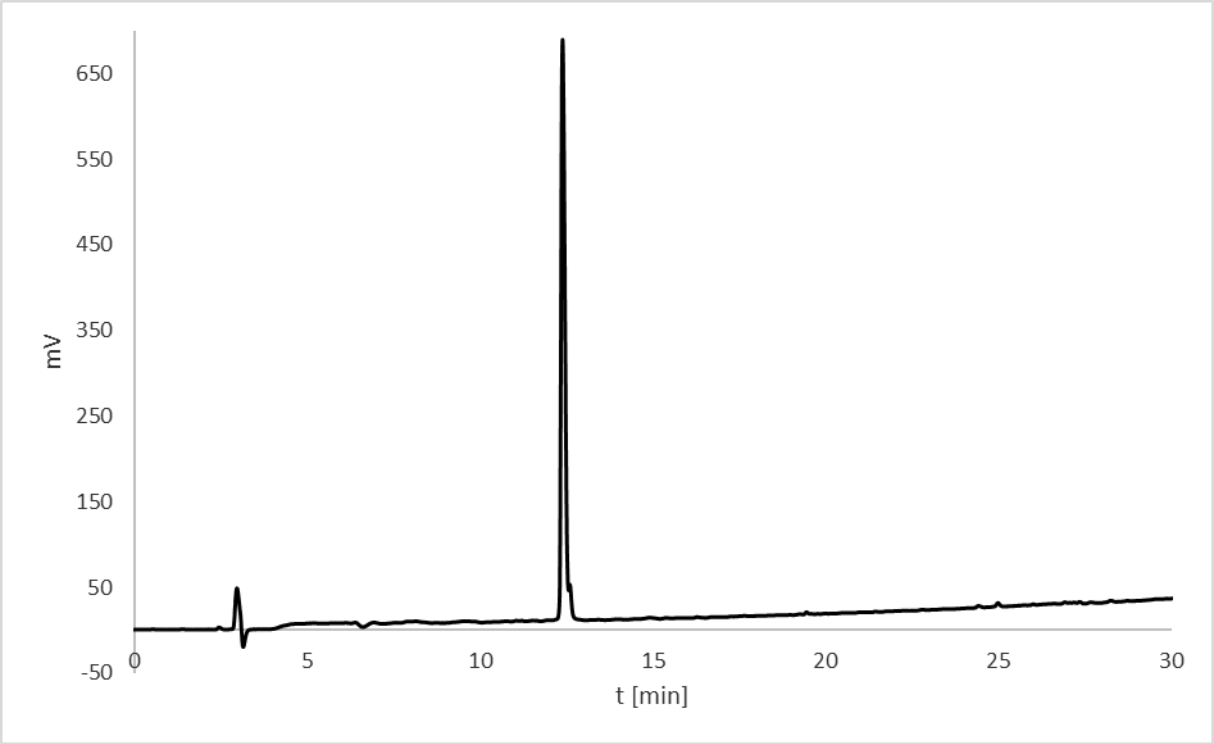
^{1}H-NMR Spectrum of Thr$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S14** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Thr$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S14** (200 MHz, [d6]-DMSO)

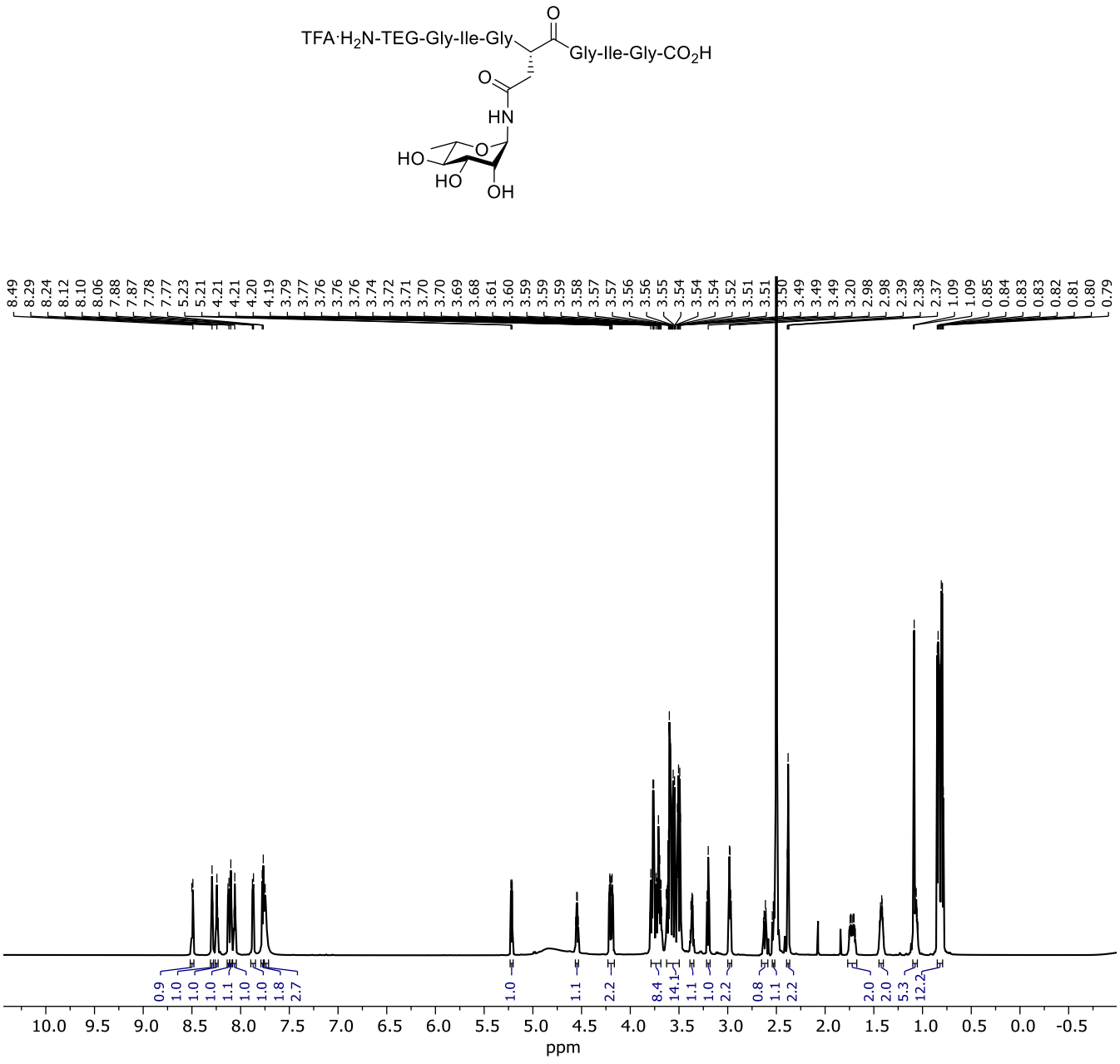


1H-13C-coupled HSQC-Spectrum of Glycopeptide **S14**: H-1,C-1 coupling constant JH1,C1

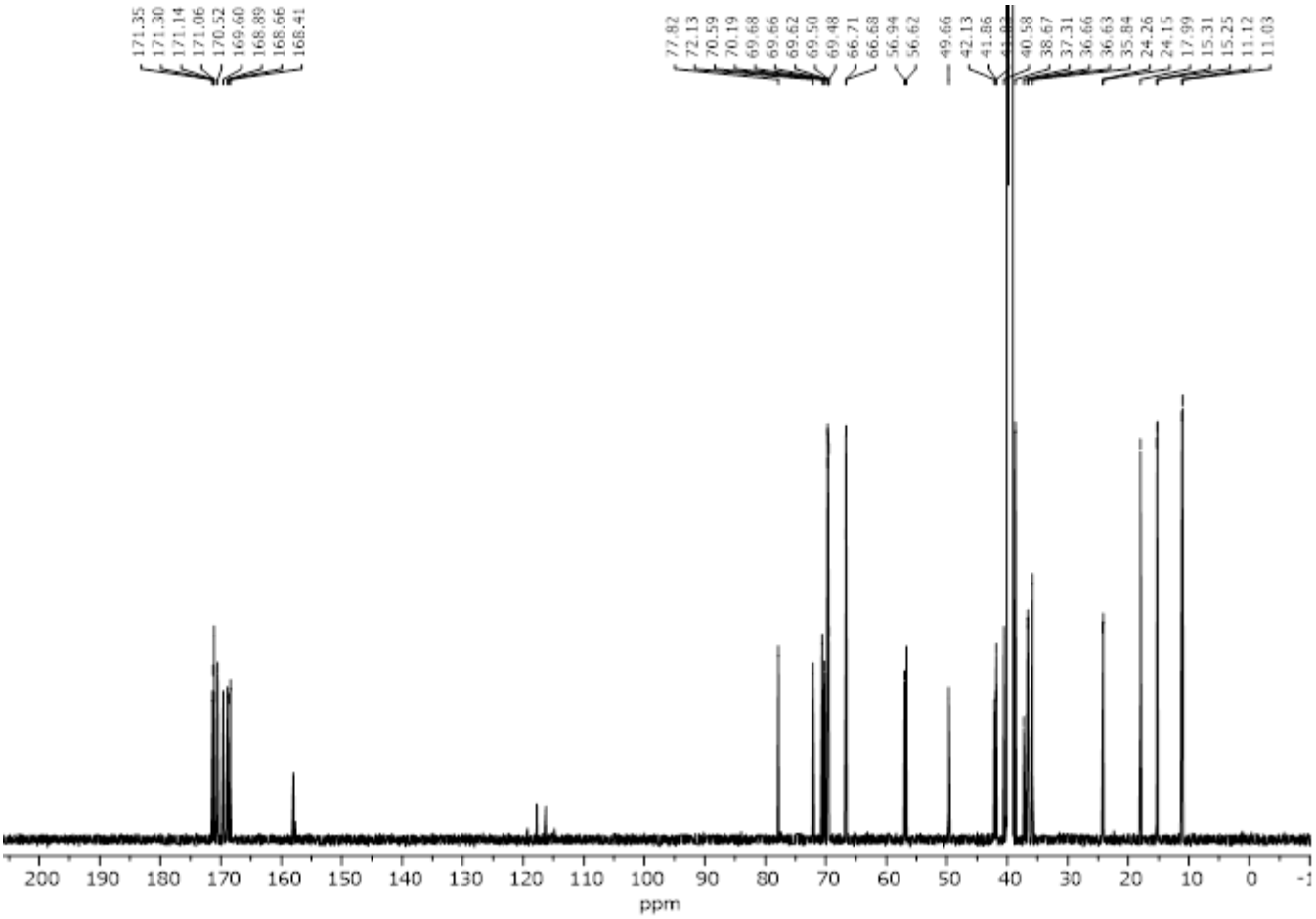


Analytical HPLC-trace of Thr$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S14**

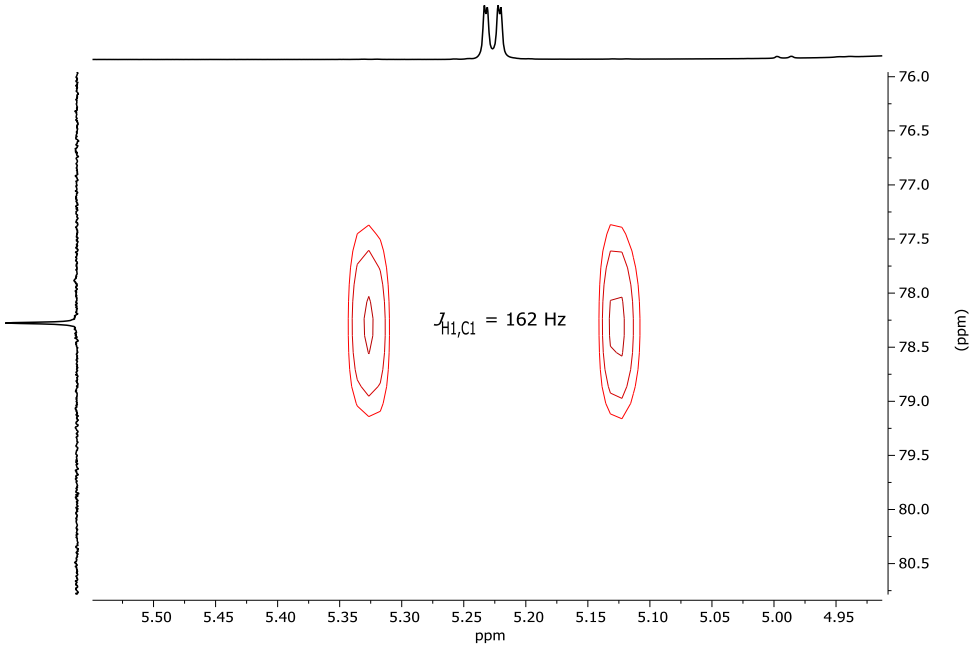
Asn$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **(S15)**



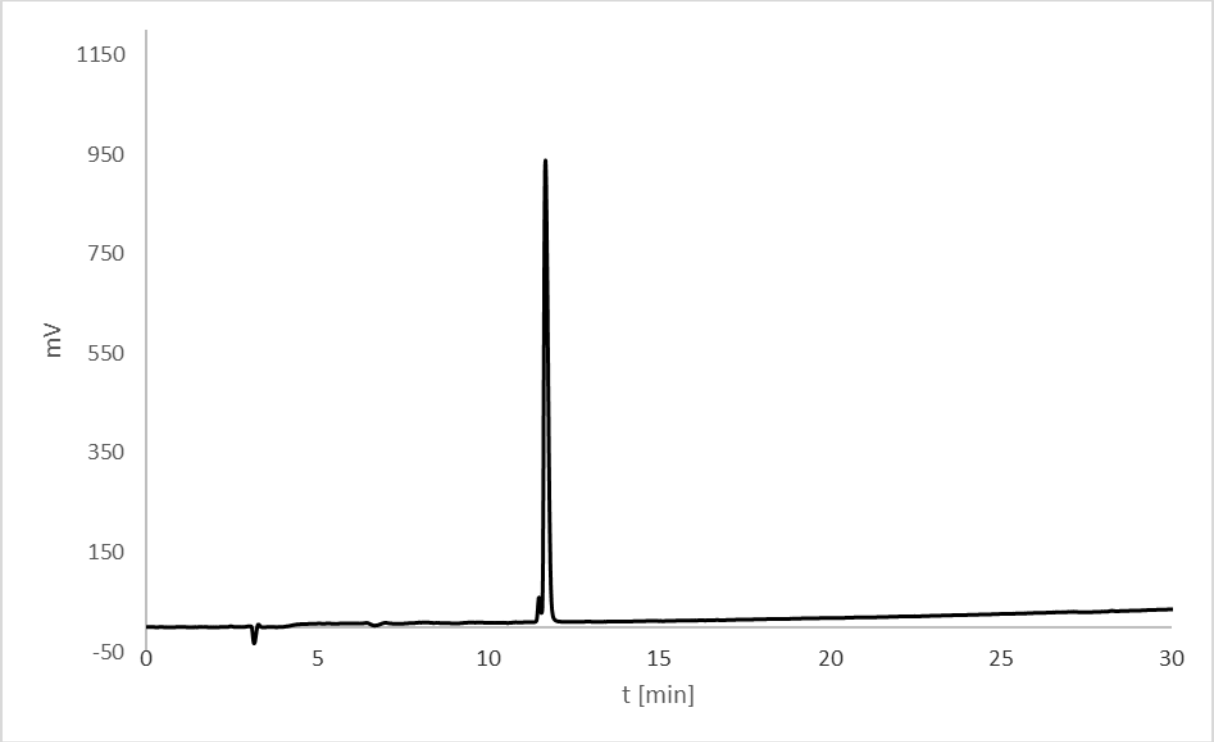
^{1}H-NMR Spectrum of Asn$^{\alpha\text{-Rha}}$-Peptide **S15** (800 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Asn$^{\alpha\text{-Rha}}$-Peptide **S15** (200 MHz, [d6]-DMSO)

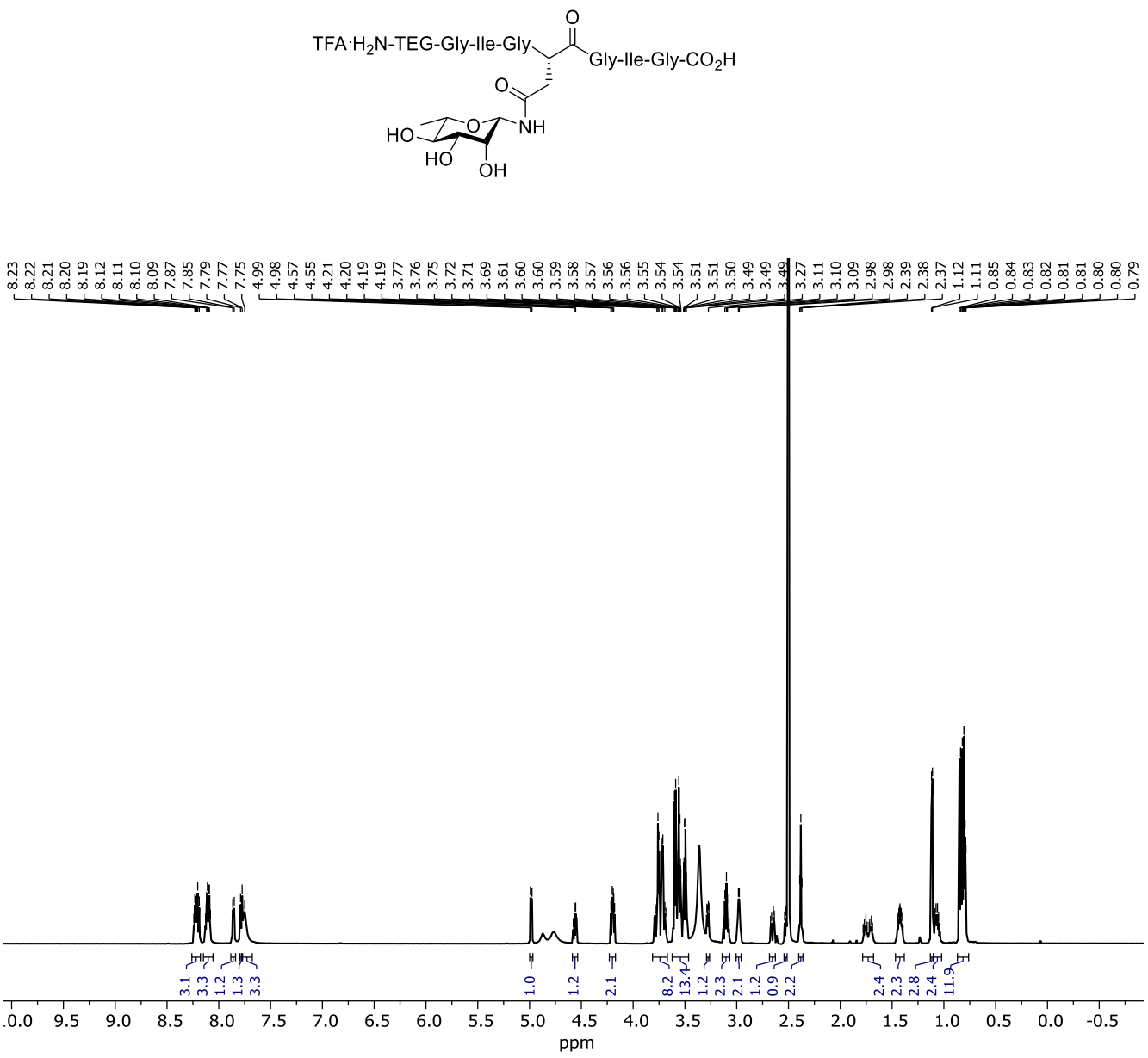


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **S15**: H-1,C-1 coupling constant $J_{H1,C1}$

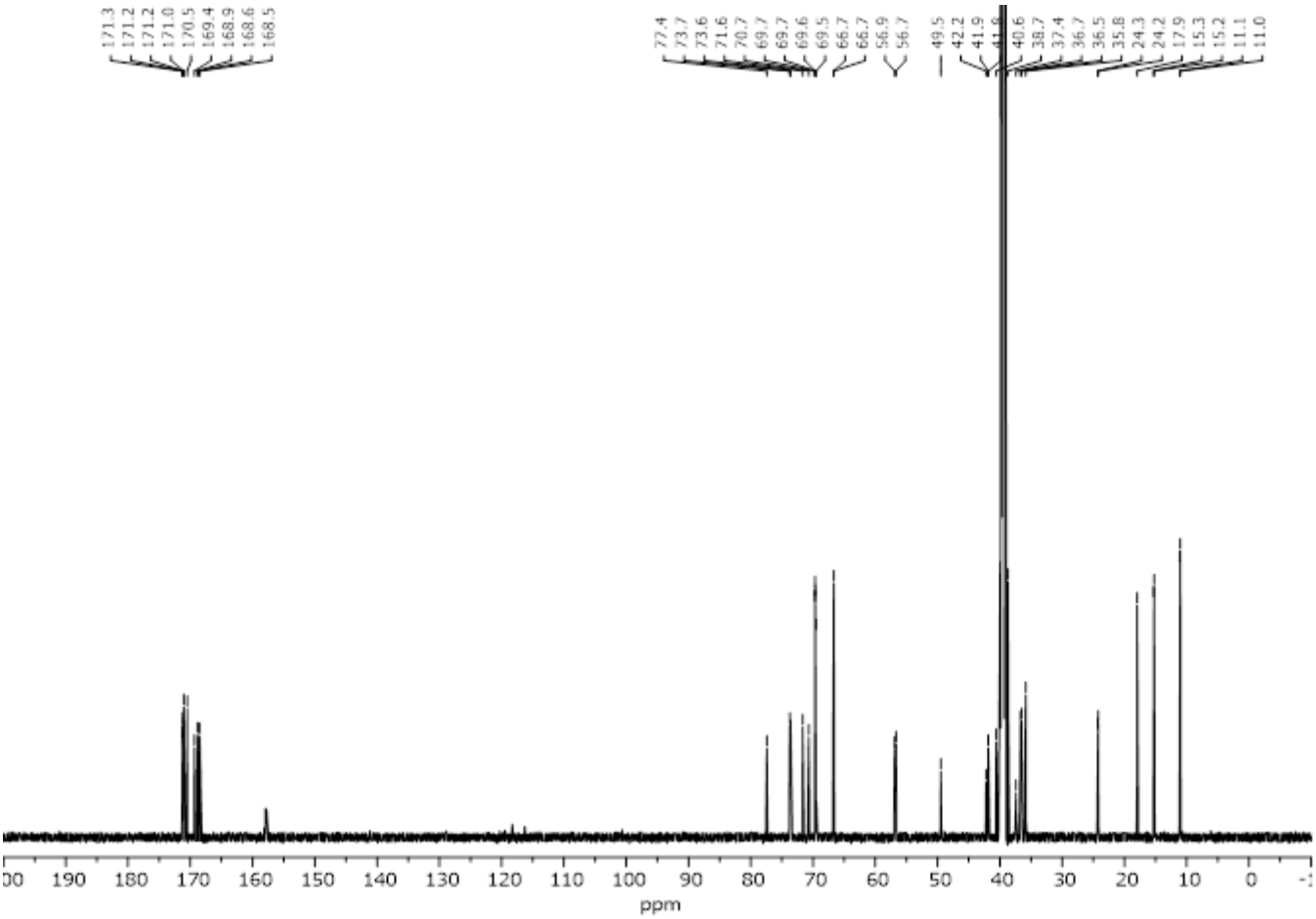


Analytical HPLC-trace of Asn$^{\alpha\text{-Rha}}$-Peptide with TEG-Spacer **S15**

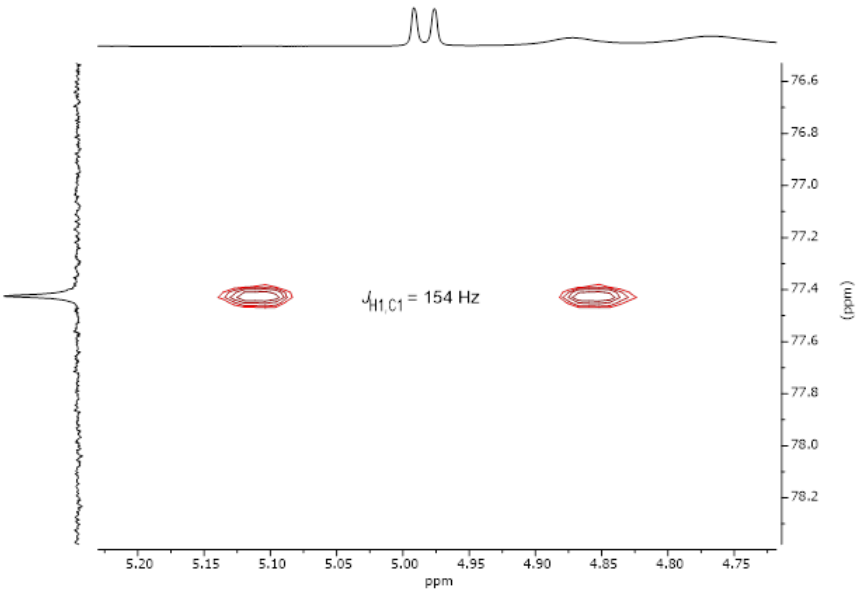
Asn$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer (**S16**)



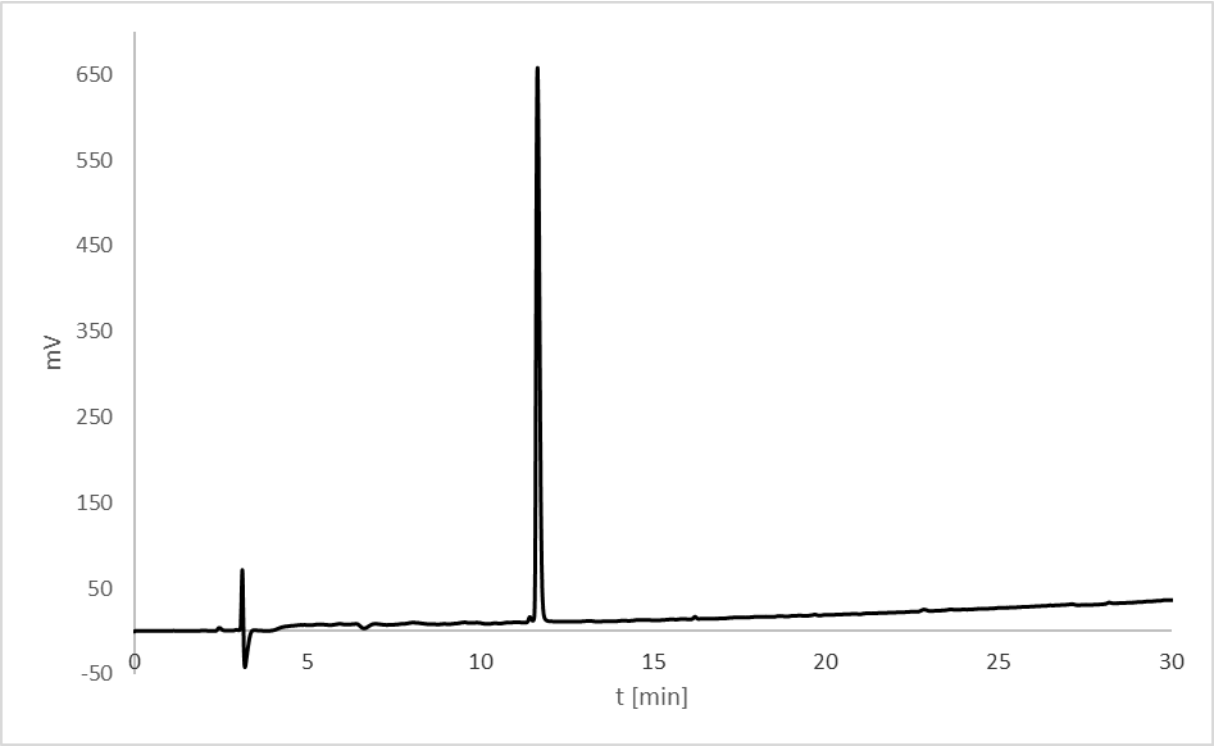
^{1}H-NMR Spectrum of Asn$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S16** (600 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Asn$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S16** (150 MHz, [d6]-DMSO)

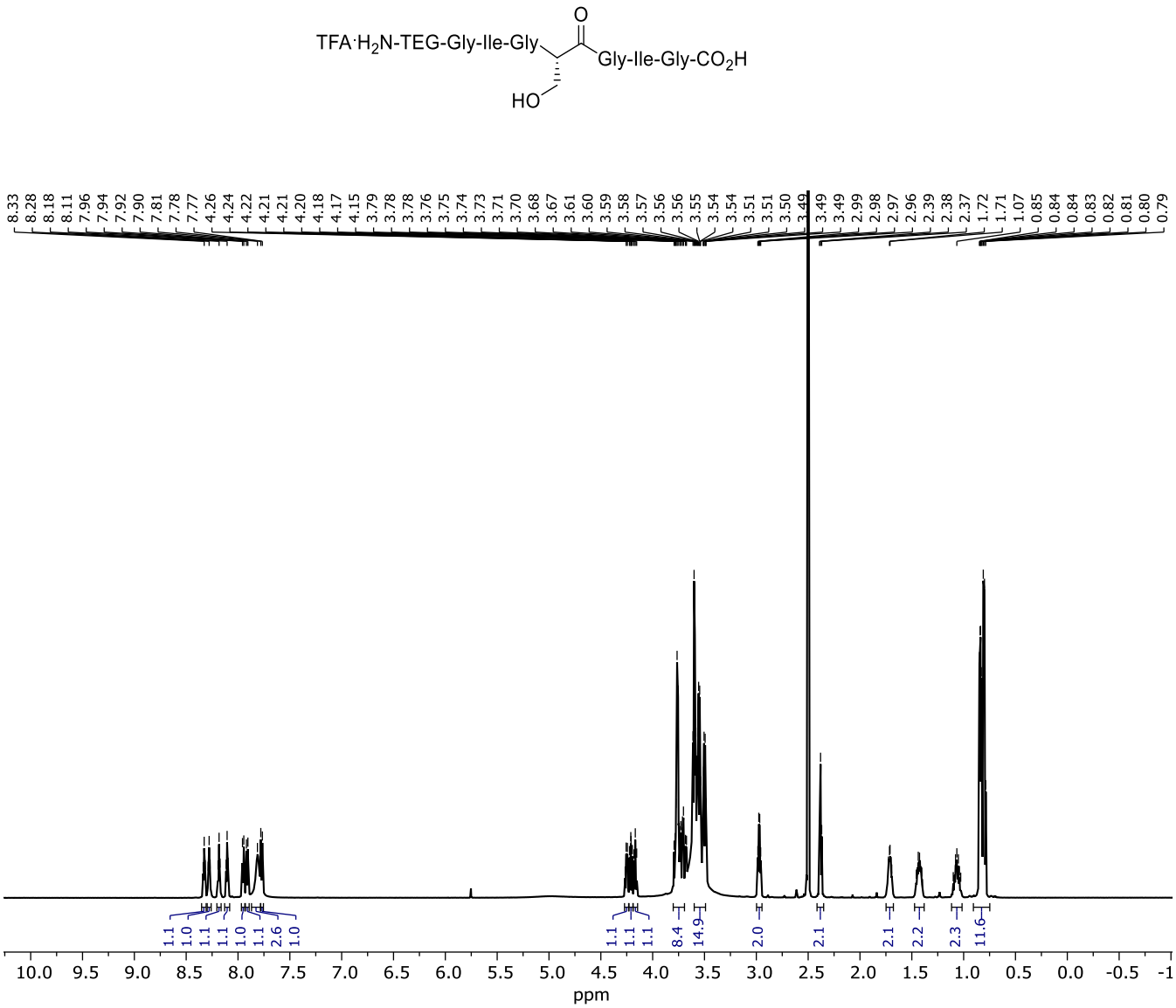


^{1}H-^{13}C-coupled HSQC-Spectrum of Glycopeptide **S16**: H-1,C-1 coupling constant $J_{H1,C1}$

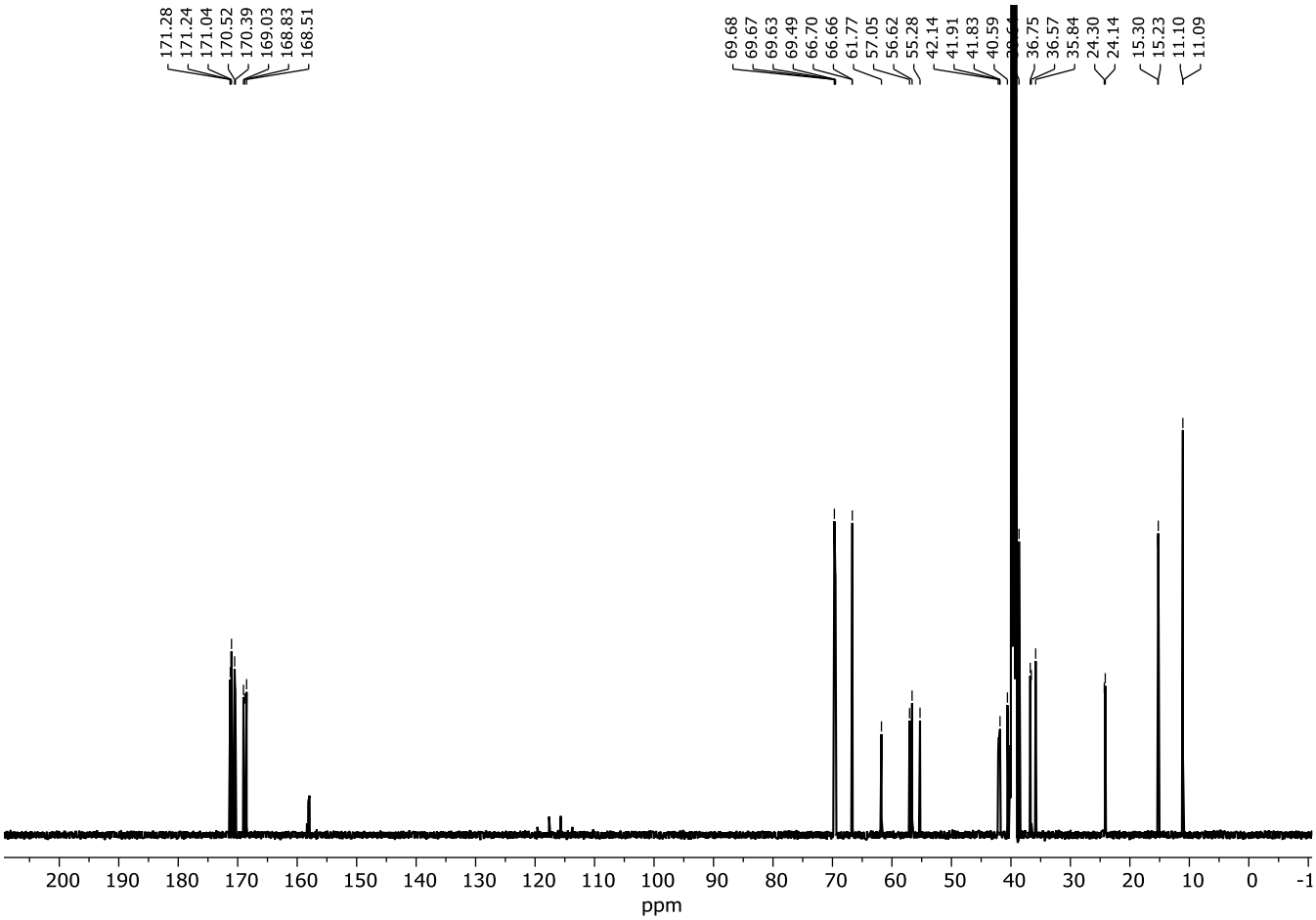


Analytical HPLC trace of Asn$^{\beta\text{-Rha}}$-Peptide with TEG-Spacer **S16**

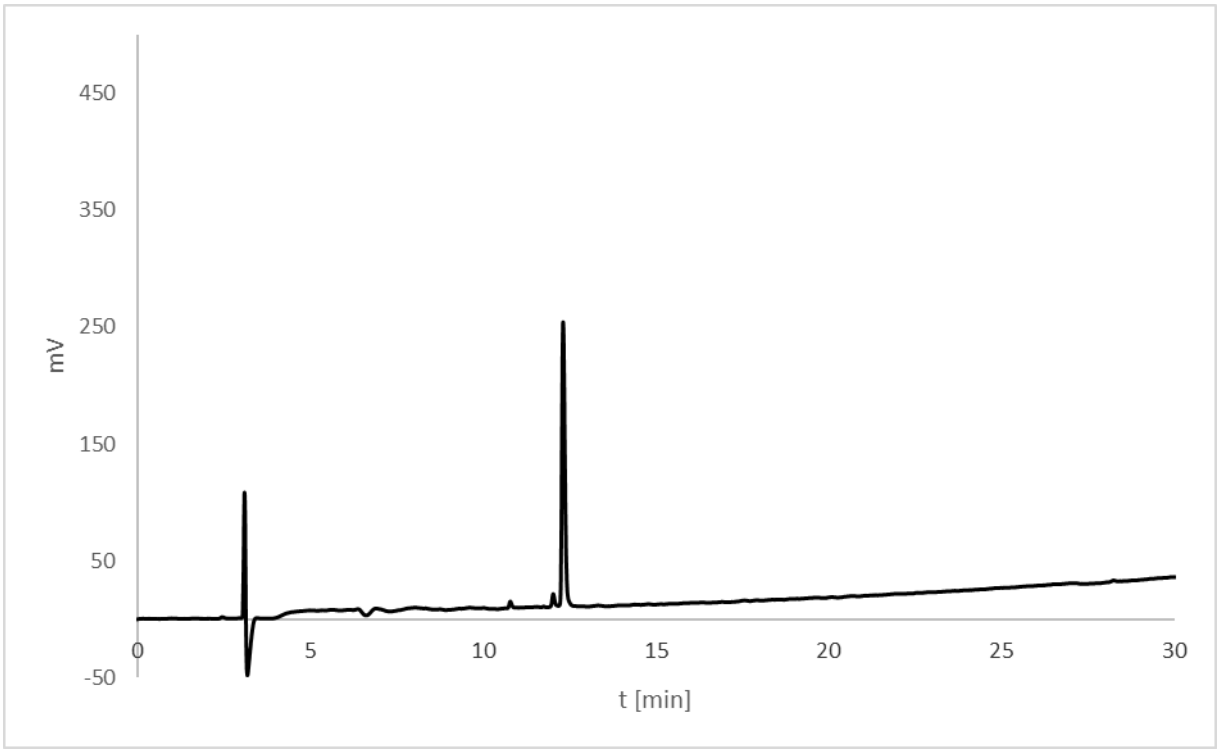
Naked Ser-Peptide (**S17)**



^{1}H-NMR Spectrum of naked Ser-Peptide with TEG-Spacer **S17** (600 MHz, [d6]-DMSO)

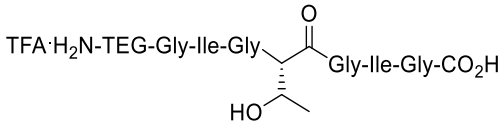


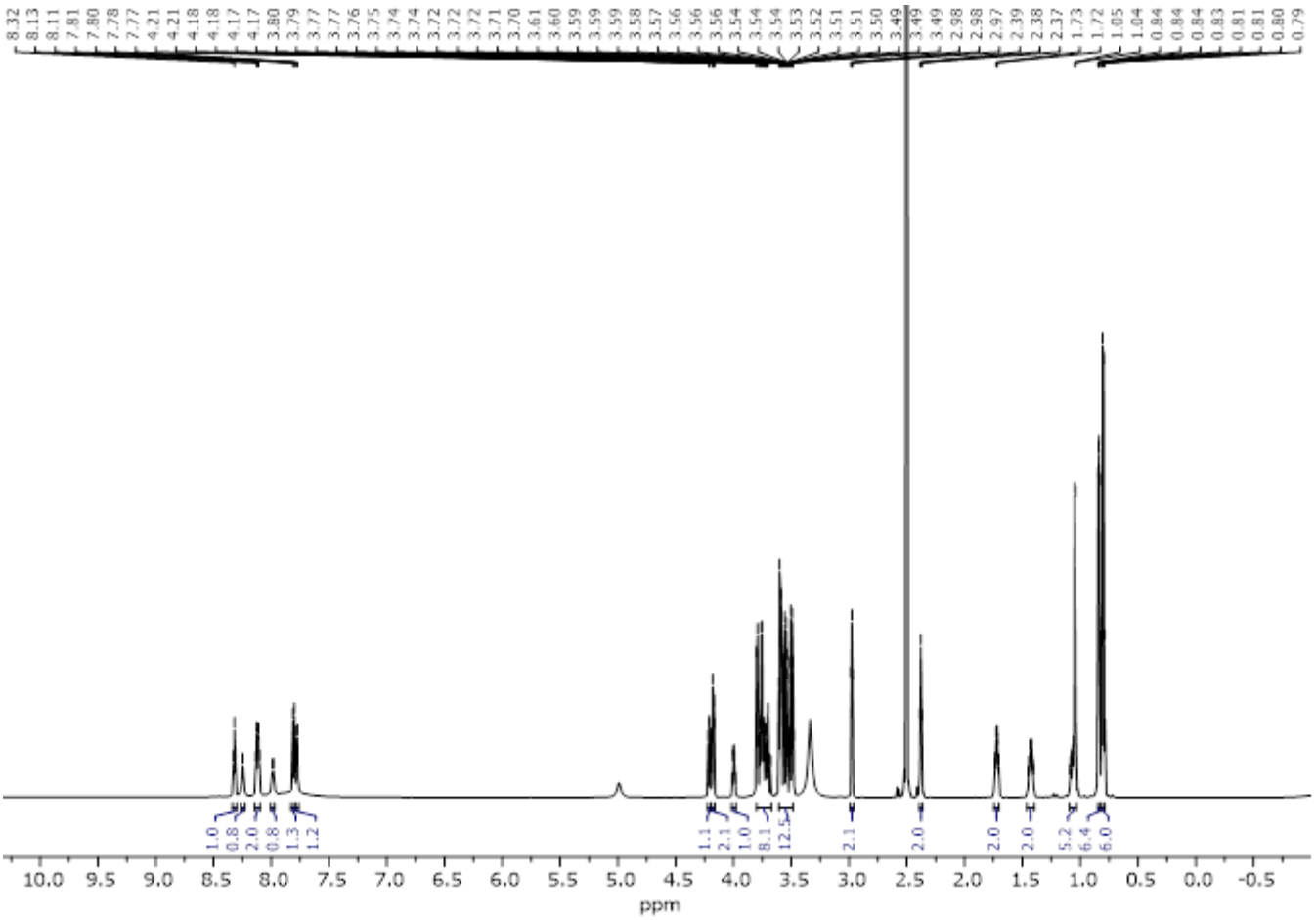
^{13}C-NMR Spectrum of naked Ser-Peptide with TEG-Spacer **S17** (150 MHz, [d6]-DMSO)



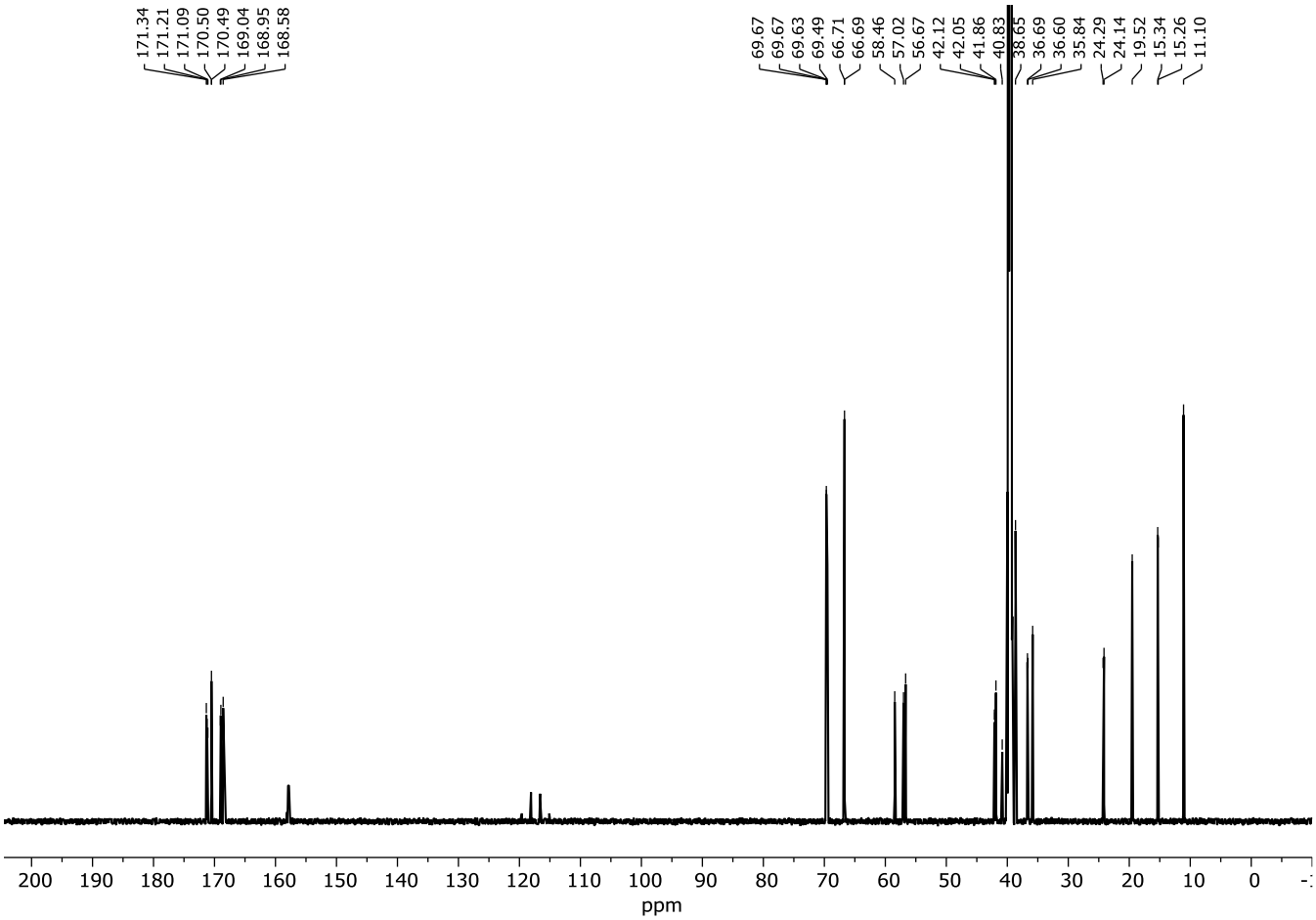
Analytical HPLC-trace of naked Ser-Peptide **S17**

Naked Thr-Peptide **S18**

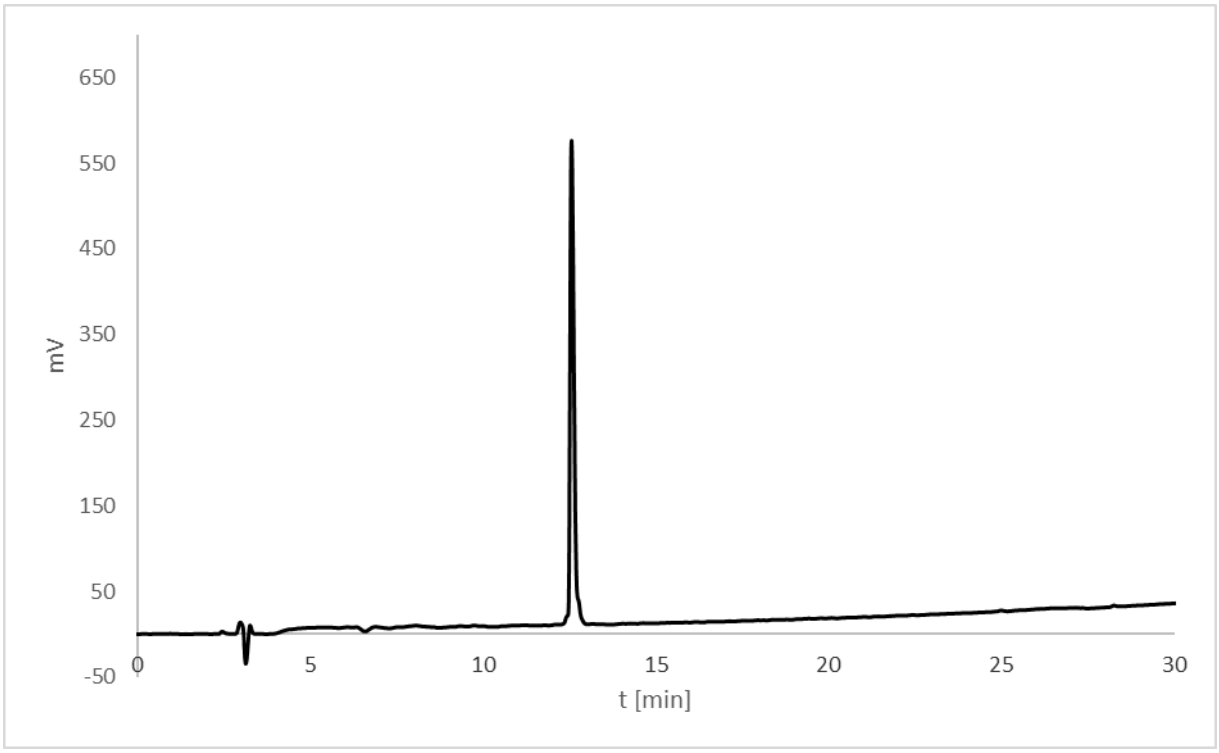




^{1}H-NMR Spectrum of naked Thr-Peptide with TEG-Spacer **S18** (800 MHz, [d6]-DMSO)

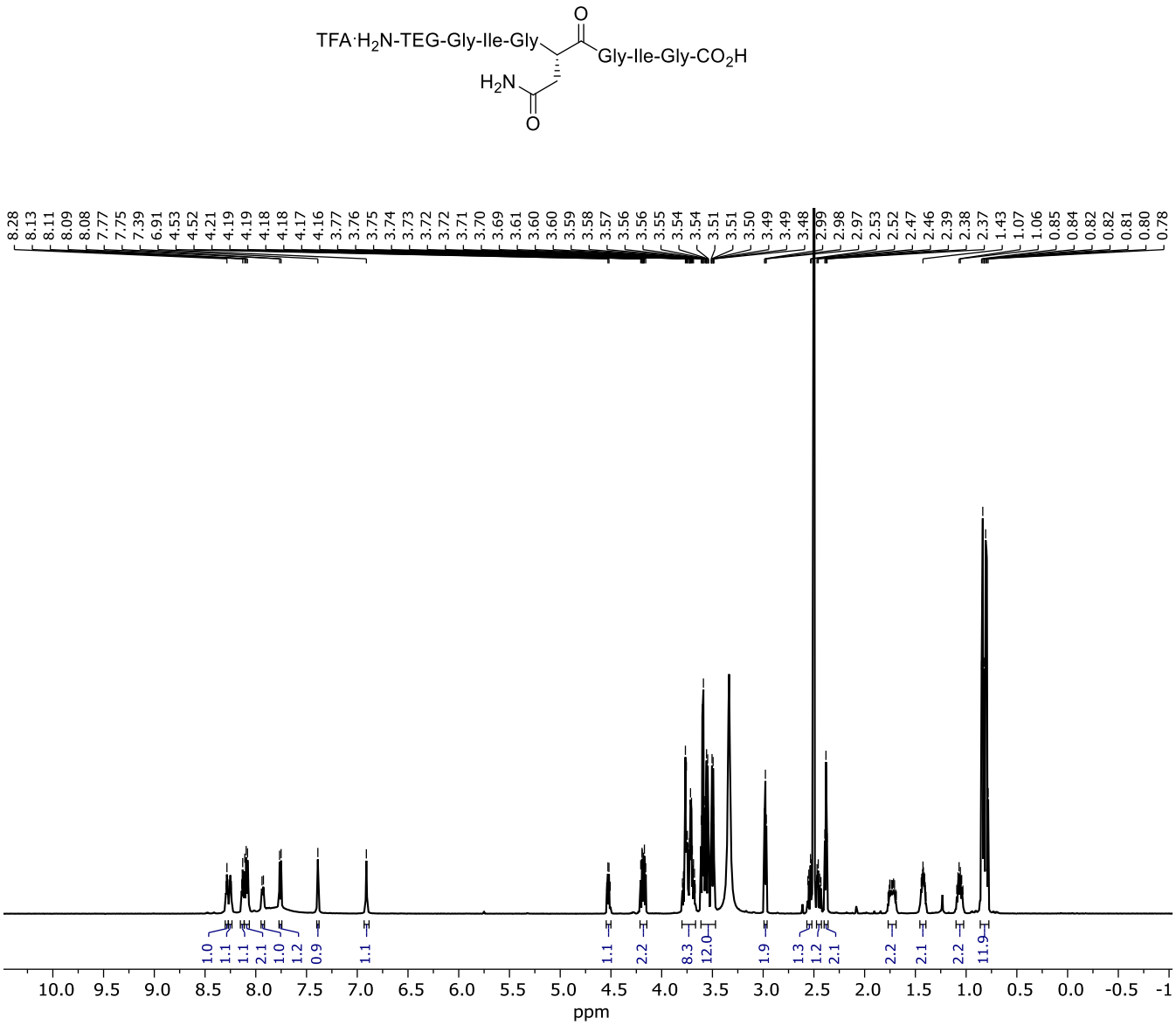


^{13}C-NMR Spectrum of naked Thr-Peptide with TEG-Spacer **S18** (200 MHz, [d6]-DMSO)

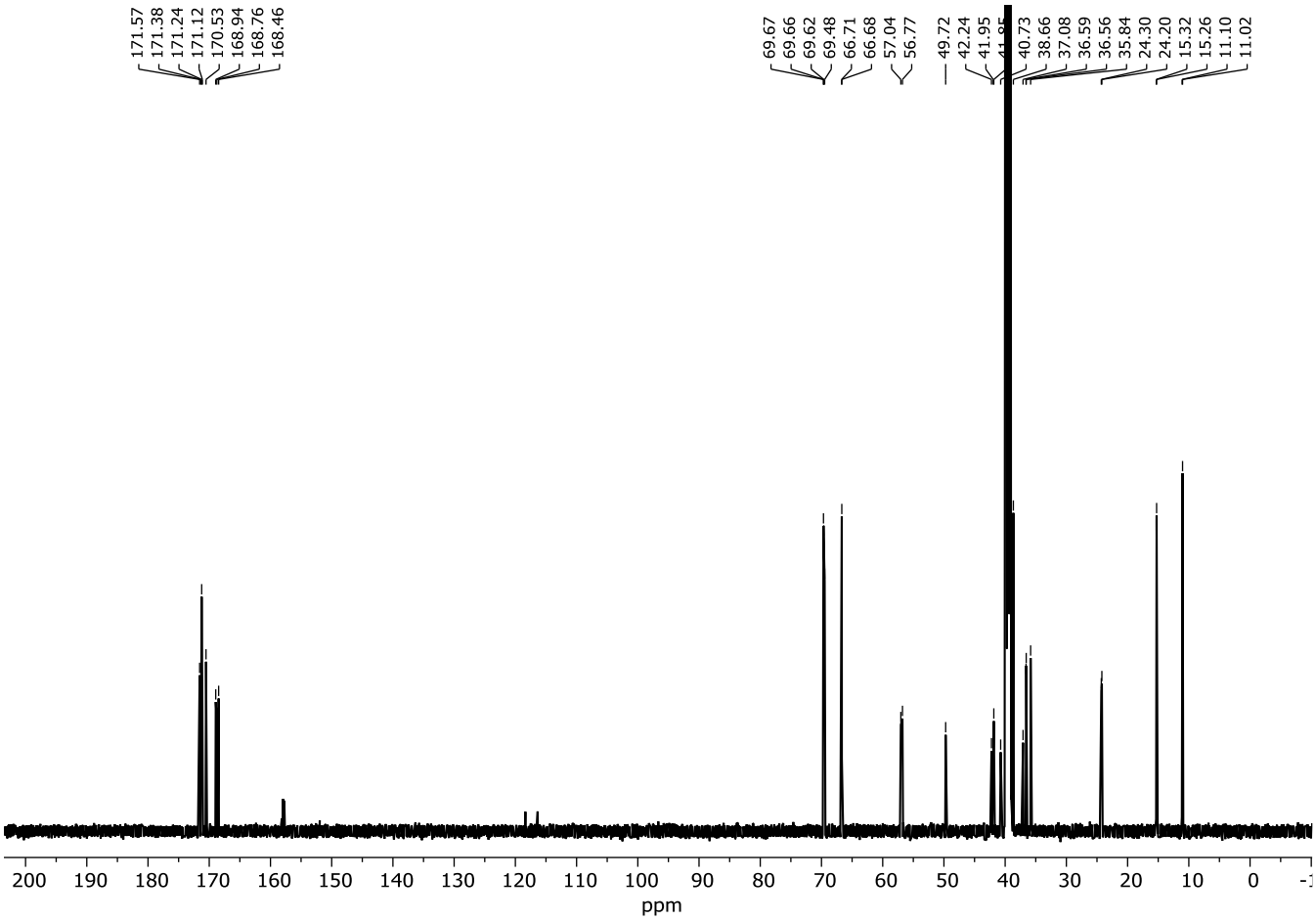


Analytical HPLC-trace of naked Thr-Peptide **S18**

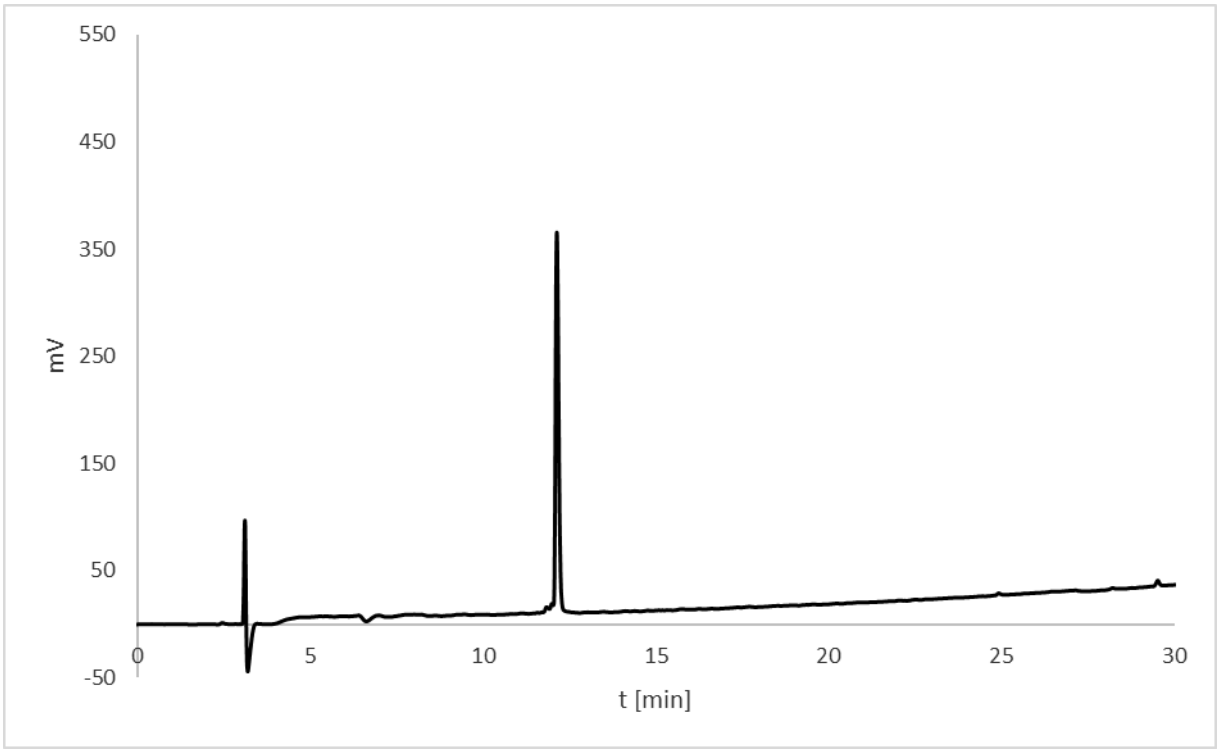
Naked Asn-Peptide (**S19**)



^{1}H-NMR Spectrum of Naked Asn-Peptide with TEG-Spacer **S19** (600 MHz, [d6]-DMSO)



^{13}C-NMR Spectrum of Naked Asn-Peptide with TEG-Spacer **S19** (150 MHz, [d6]-DMSO)



Analytical HPLC-Trace of Naked Asn-Peptide **S19**

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