

## SUPPORTING INFORMATION

### Accelerated solid-phase synthesis of glycopeptides containing multiple N-glycosylated sites

Poriah Strauss, Francesca Nuti, Michael Quagliata, Anna Maria Papini and Mattan Hurevich

<sup>a</sup> The Institute of Chemistry, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram, Jerusalem, 91904, Israel

<sup>b</sup> Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry “Ugo Schiff”, University of Florence, 50019 Sesto Fiorentino, Italy

\*Corresponding authors. Email: [mattan.hurevich@mail.huji.ac.il](mailto:mattan.hurevich@mail.huji.ac.il) ; [annamaria.papini@unifi.it](mailto:annamaria.papini@unifi.it)

#### 1. Instruments

##### 1.1 Analytical HPLC

HPLC analyses were performed on a Merck Hitachi system equipped with an L-2130 pump, L-2400 UV detector, and an XTerra RP8 column (125Å, 5 µm, 4.6 mm X 250 mm). All samples were dissolved in TDW/ACN 50:50, filtered through 0.22 µm PTFE filters, and injected into a reversed-phase analytical HPLC column (RP-HPLC). The mobile phase consisted of solution A: TDW (0.1% v/v TFA) and solution B: ACN (0.1% v/v TFA). The glycopeptides were analyzed using gradient 5:95. Chromatograms were recorded at 220 nm at room temperature with a flow rate of 1 mL/min. Glycopeptides were analyzed with a gradient from 5:95 to 95:5 (B:A) in 20 min. The collected fractions were analyzed by ESI-MS. The crude purity of each glycopeptide was calculated by integration of the specific peak that was detected by ESI-MS.

Table S1 Analytical HPLC gradient program

Time	A (TDW)	B (ACN)
0	95	5
5	95	5
25	5	95
30	5	95

##### 1.2 Preparative HPLC

Crude glycopeptides were purified on a Waters HPLC system with a 2545 Binary Gradient Module and a 2849 UV detector (220 nm) using a Phenomenex Luna C18 HPLC column (5 µm, 250 x 21.2 mm). Each crude sample after lyophilization was dissolved in 4.5 mL of TDW/ACN 50:50 and injected into the reversed-phase preparative HPLC column. The preparative HPLC was performed with a B:A gradient 5:95 to 60:40 in 35 min at room temperature with a flow rate of 15 mL/min. The mobile phase consisted of solution A: TDW

(0.1% v/v TFA) and solution B: ACN (0.1% v/v TFA). Chromatograms were recorded at 220 nm at room temperature.

Table S2 Preparative HPLC gradient program

Time	A (TDW)	B (ACN)
0	95	5
5	95	5
35	40	60
42	5	95

### 1.3 ESI-MS

ESI-MS were performed on LCQ Fleet Ion Trap mass spectrometer (Thermo Fischer Scientific, San Diego, CA, USA). Masses of the glycopeptides were calculated as experimental mass ratios (m/z) of the observed multiply charged species of each glycopeptide. Deconvolution of the MS data were performed using MagTran v1.03 software.

### 1.4 Calculation of the yield of crude and purified peptides

The yield was determined based on the following equation (Eq. S1).

$$Eq. S1: Yield = \frac{Peptide\ weight}{Calculated\ peptide\ weight} * 100\%$$

$$Yield = \frac{Peptide\ weight}{Resin\ weight * loading * Peptide\ molar\ weight} * 100\%$$

The weight was measured using the analytical balance: A/D HR-120 (Max. 120 gr., d = 0.1 mg).

## 2 HPLC and mass spectrometry analyses of crude and purified N-glycosylated peptides

### GP-1 synthesized on Rink Amide MBHA resin

Sequence: VTLN[Glc(OAc)<sub>4</sub>]TTGTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1352</sup>(Glc)]HMW1ct(1349-1357)

Resin: Rink Amide MBHA (loading 0.48 mmol/g)

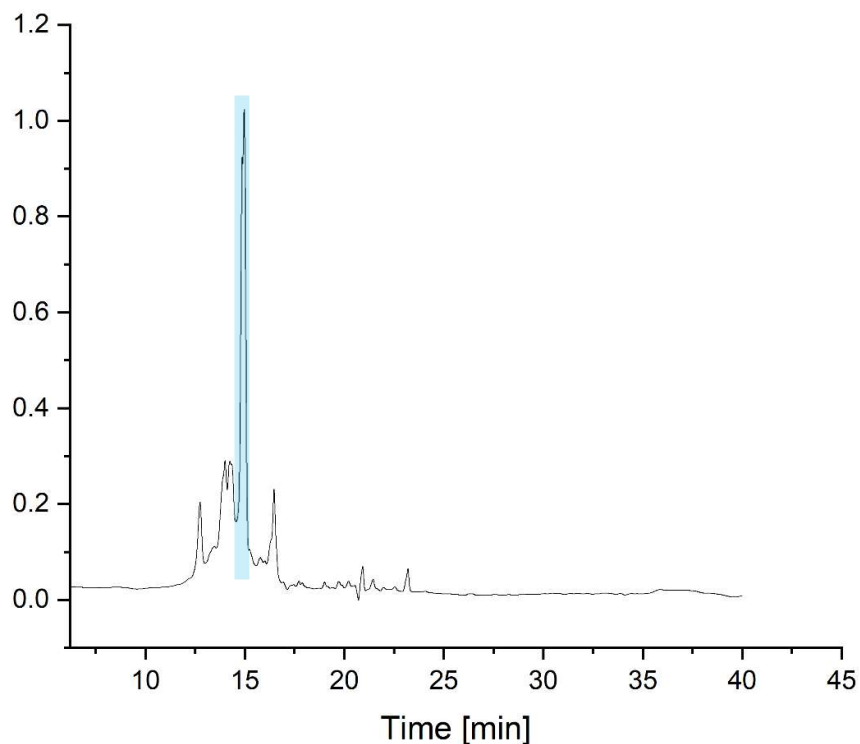


Figure S1 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-1** (peak highlighted in blue) synthesized on Rink Amide MBHA resin (loading 0.48 mmol/g).

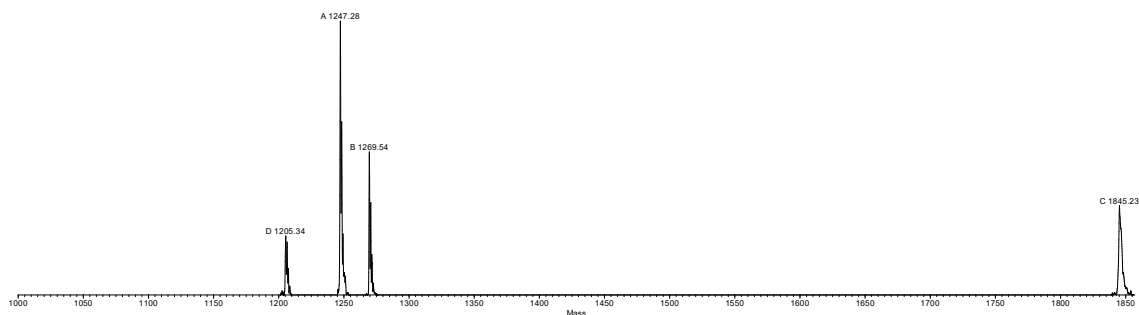


Figure S2 – ESI-MS analysis of **GP-1**.

**Peak A:** **GP-1**, C<sub>53</sub>H<sub>88</sub>N<sub>11</sub>O<sub>23</sub>, Exact mass calcd.: 1247.32; found: 1247.28.

**Peak D:** **Des-Ac-GP-1**, C<sub>51</sub>H<sub>86</sub>N<sub>11</sub>O<sub>22</sub>, Exact mass calcd.: 1205.61; found: 1205.34.

**GP-2 synthesized on Rink Amide MBHA resin**

Sequence: N[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1348-1357)

Resin: Rink Amide MBHA (loading 0.48 mmol/g)

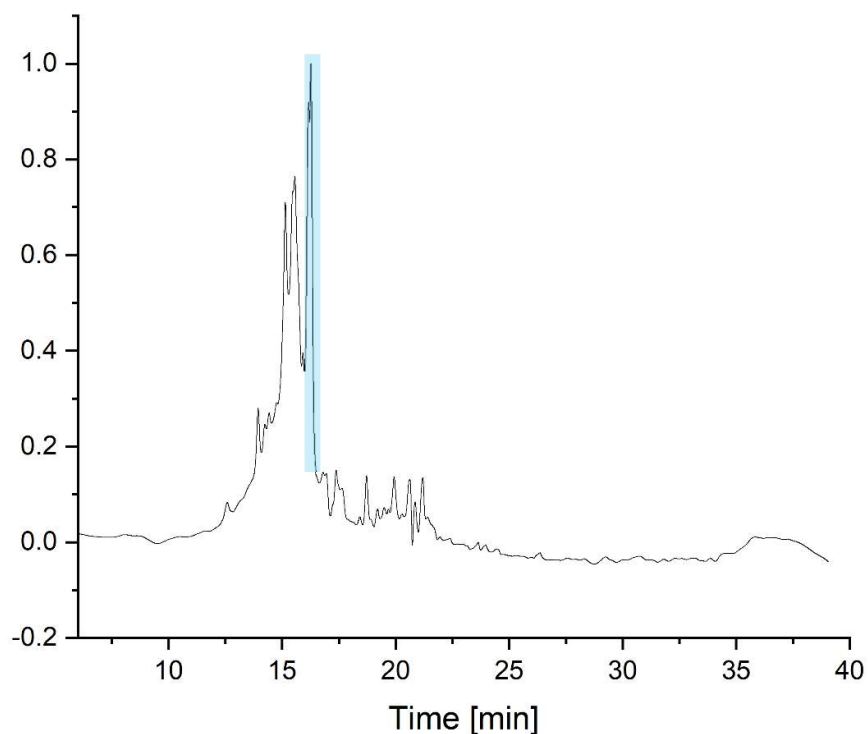


Figure S3 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-2** (peak highlighted in blue) synthesized on Rink Amide MBHA resin (loading 0.48 mmol/g).

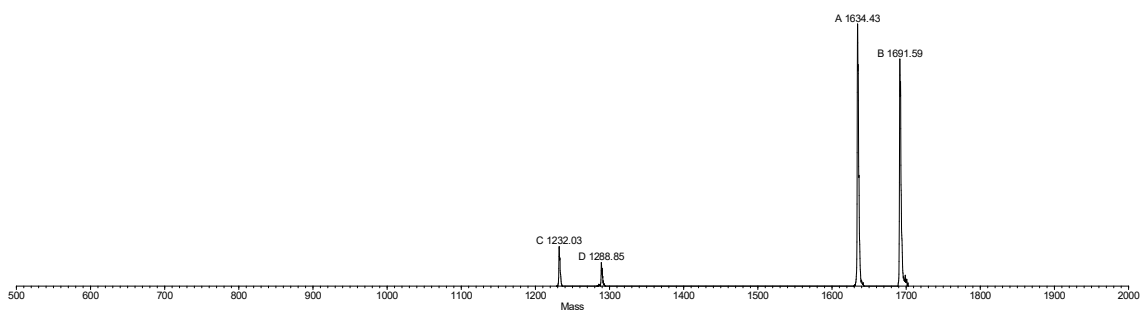


Figure S4 – ESI-MS analysis of **GP-2**.

**Peak A:** Des-Gly<sup>1355</sup>GP-2, C<sub>69</sub>H<sub>110</sub>N<sub>12</sub>O<sub>33</sub>, Exact mass calcd.: 1635.67; found: 1634.43.

**Peak B:** GP-2, N[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>, C<sub>71</sub>H<sub>112</sub>N<sub>13</sub>O<sub>34</sub>, Exact mass calcd.: 1691.75; found: 1691.59

**GP-3 synthesized on Rink Amide MBHA resin**

Sequence: AN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1347-1357)

Resin: Rink Amide MBHA (loading 0.48 mmol/g)

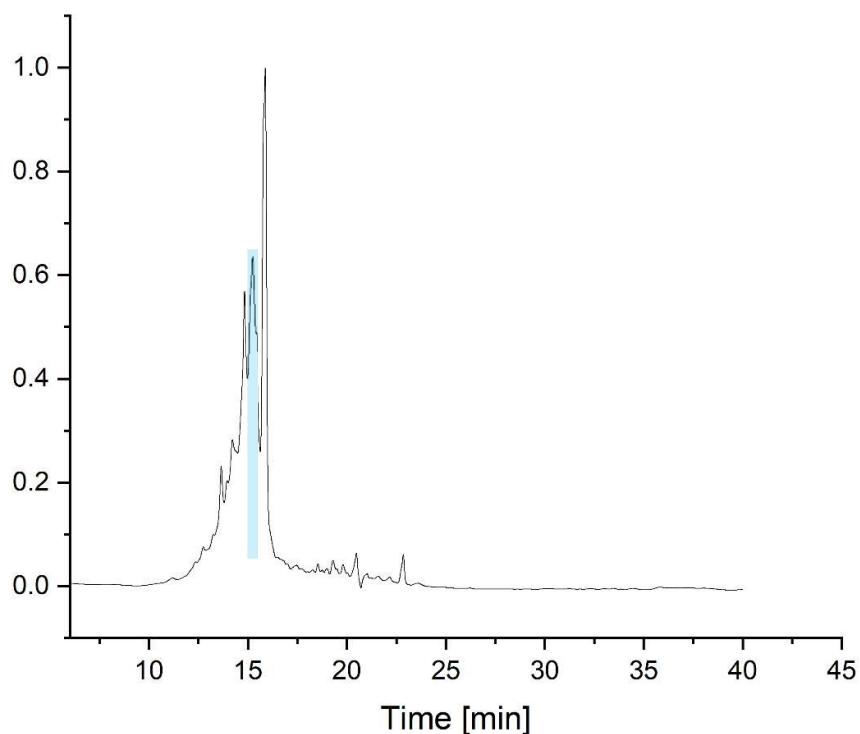


Figure S5 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-3** (peak highlighted in blue) synthesized on Rink Amide MBHA resin (loading 0.48 mmol/g).

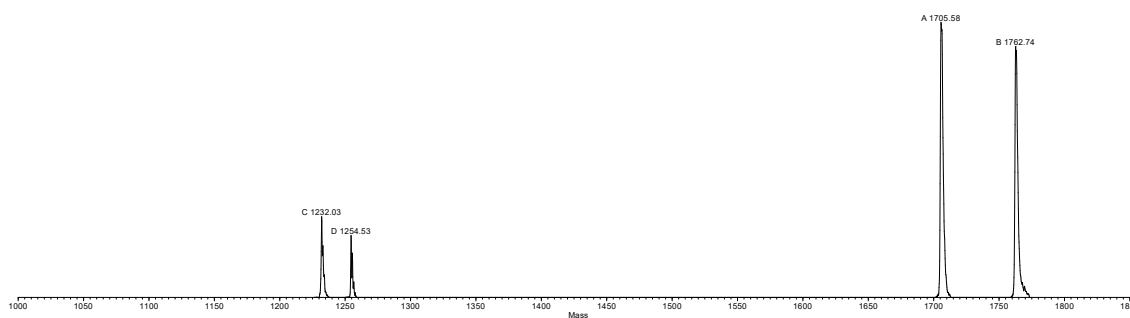


Figure S6 – ESI-MS analysis of **GP-3**.

**Peak A:** Des-Gly<sup>1355</sup>-GP3, C<sub>72</sub>H<sub>115</sub>N<sub>13</sub>O<sub>34</sub>, Exact mass calcd.: 1706.75; found: 1705.56.

**Peak B:** GP-3, AN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTGTL-NH<sub>2</sub>, C<sub>74</sub>H<sub>117</sub>N<sub>14</sub>O<sub>35</sub>, Exact mass calcd.: 1762.79; found: 1762.74.

**GP-4 synthesized on Rink Amide MBHA resin**

Sequence: Y\*NAAN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>

Glc-protected precursor of [Y<sup>1344</sup>,N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1344-1357)

Resin: Rink Amide MBHA (loading 0.48 mmol/g)

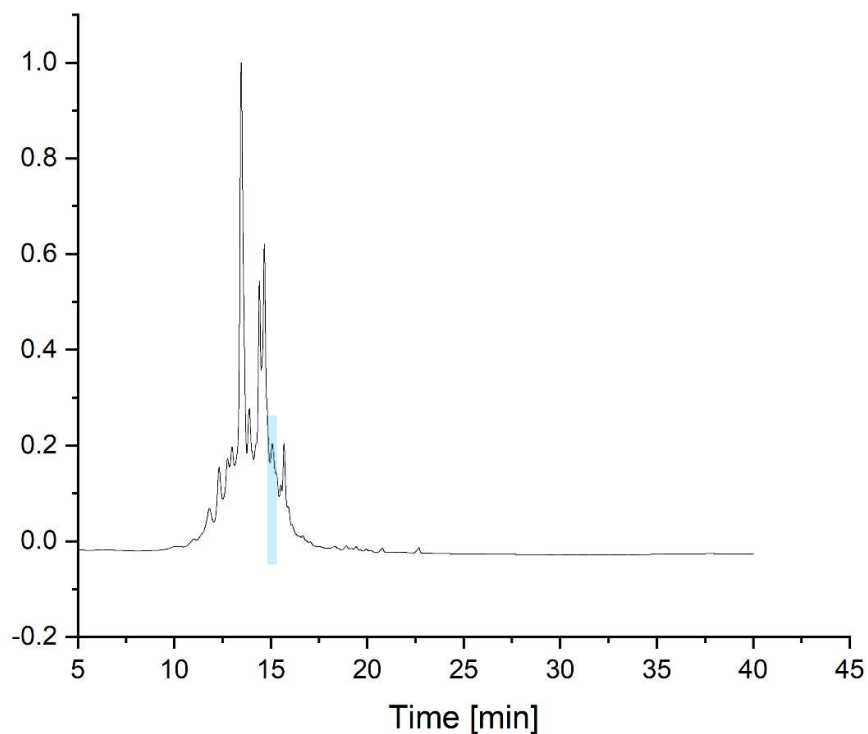


Figure S7 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-4** (peak highlighted in blue) synthesized on Rink Amide resin loading 0.48 mmol/g.

\*Tyr was introduced at the N-terminus to improve UV-HPLC detection.

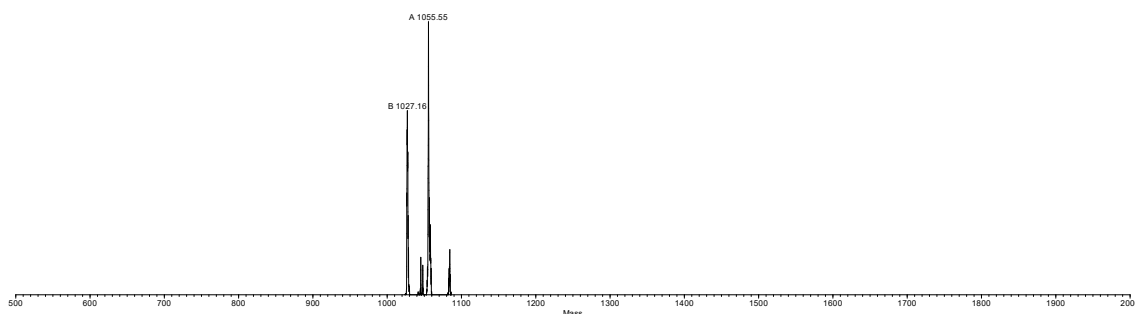


Figure S8 – ESI-MS analysis of **GP-4**.

**Peak A:** **GP-4**, Y\*NAAN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>, C<sub>90</sub>H<sub>138</sub>N<sub>18</sub>O<sub>40</sub>, Exact mass calcd.: 2110.93; found: 1055.55

**Peak B:** **Des-Gly<sup>1355</sup>GP-4**, C<sub>88</sub>H<sub>136</sub>N<sub>17</sub>O<sub>39</sub>, Exact mass calcd.: 2053.89; found: 1027.16.

**GP-2 synthesized on TentaGel R RAM resin**

Sequence: N[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1348-1357)

Resin: TentaGel R RAM (loading 0.18 mmol/g)

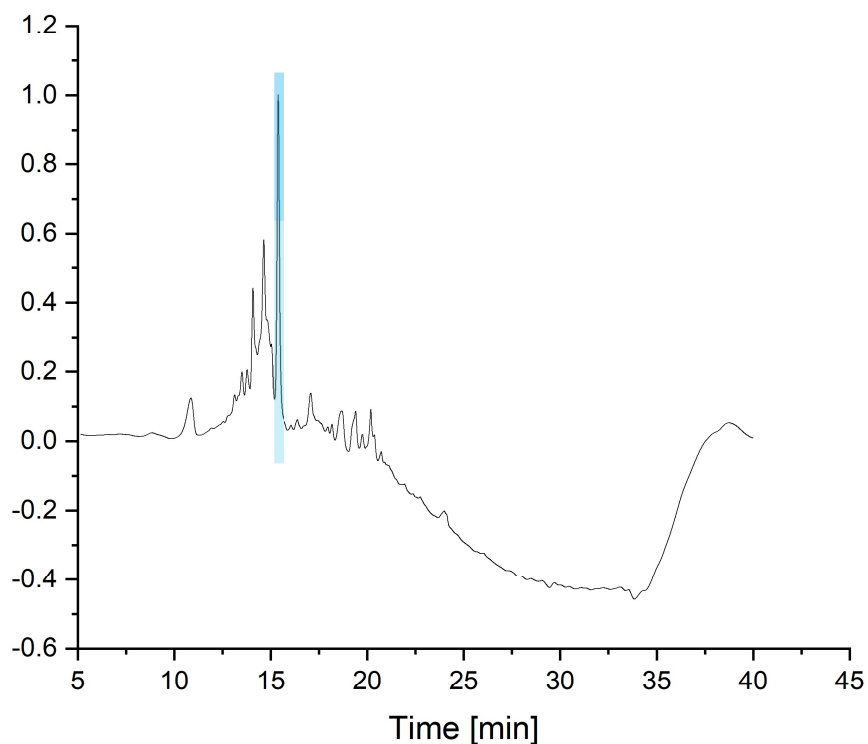


Figure S9 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-2** (peak highlighted in blue) synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

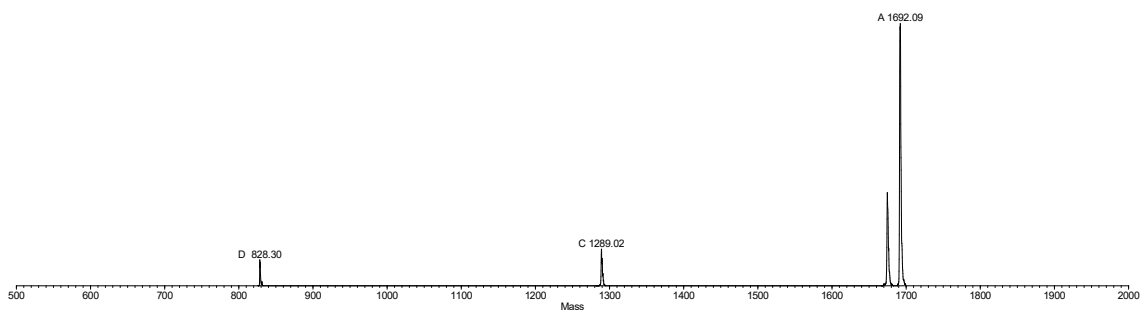


Figure S10 – ESI-MS analysis of **GP-2**.

**Peak A:** **GP2**, N[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>, C<sub>71</sub>H<sub>112</sub>N<sub>13</sub>O<sub>34</sub>, Exact mass calcd.: 1691.75; found: 1692.09.

**GP-3 synthesized on TentaGel R RAM resin**

Sequence: AN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1347-1357)

Resin: TentaGel R RAM (loading 0.18 mmol/g)

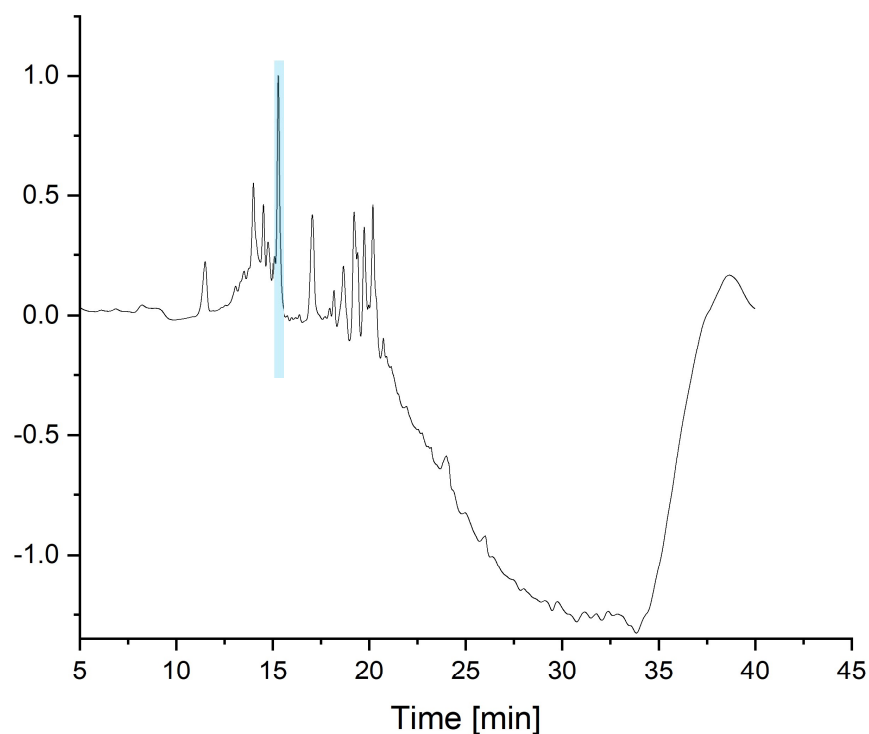


Figure S11 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-3** (peak highlighted in blue) synthesized on TentaGel R RAM (loading 0.18 mmol/g).

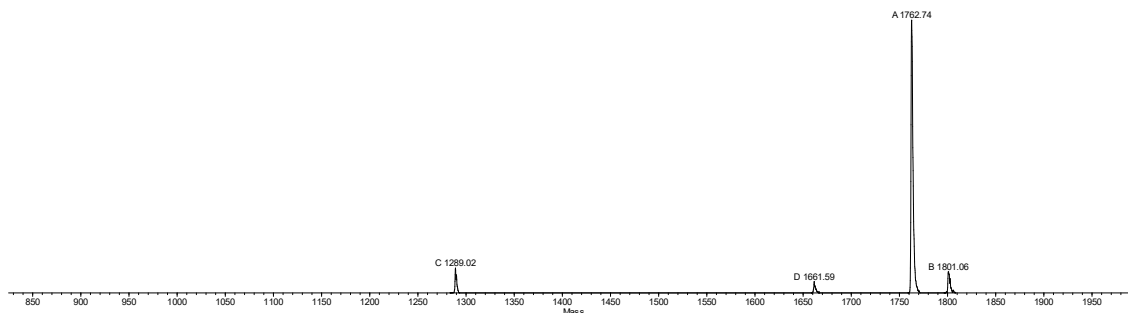


Figure S12 – ESI-MS analysis of **GP-3**.

**Peak A:** **GP-3**, AN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>, C<sub>74</sub>H<sub>118</sub>N<sub>14</sub>O<sub>35</sub>, Exact mass calcd.: 1762.78; found: 1762.74.

**Peak D:** **Des-Thr-GP-3**, C<sub>70</sub>H<sub>111</sub>N<sub>13</sub>O<sub>33</sub>, Exact mass calcd.: 1661.79; found: 1661.59.



**GP-4 synthesized on TentaGel R RAM resin**

Sequence:

Y\*NAAN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1348</sup>(Glc),N<sup>1352</sup>(Glc)]HMW1ct(1344-1357)

Resin: TentaGel R RAM (loading 0.18 mmol/g)

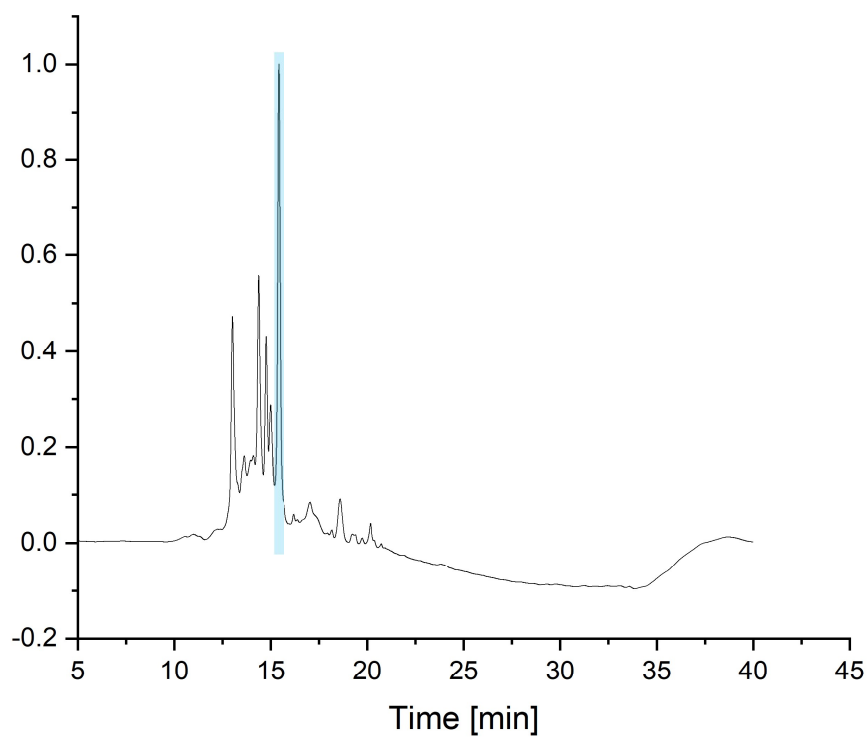


Figure S13 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-4** (peak highlighted in blue) synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

\*Tyr was introduced at the N-terminus to improve UV-HPLC detection.

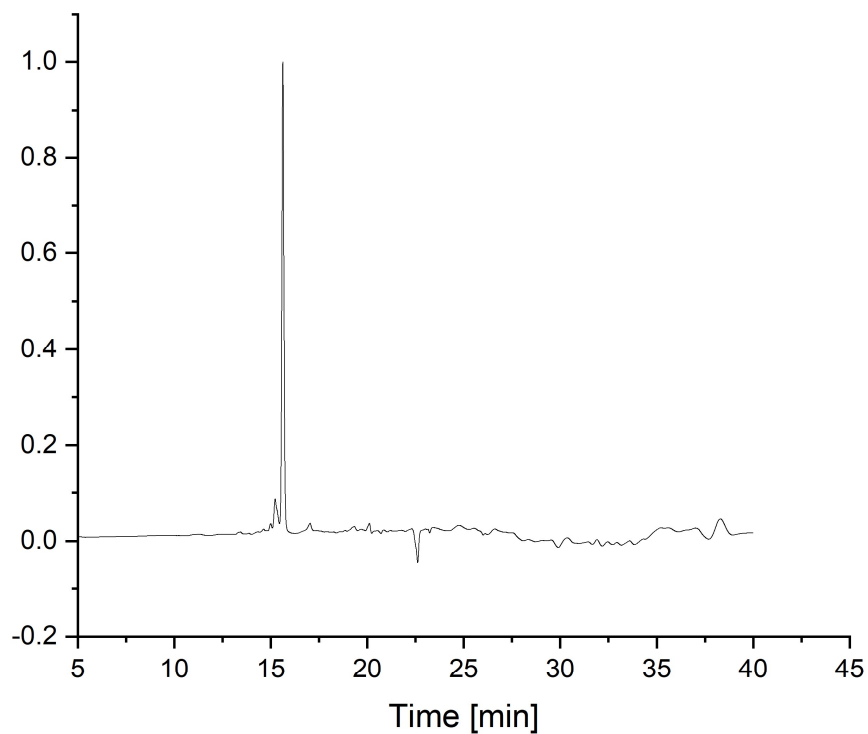


Figure S14 – Analytical HPLC chromatogram (recorded at 220 nm) of purified **GP-4** synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

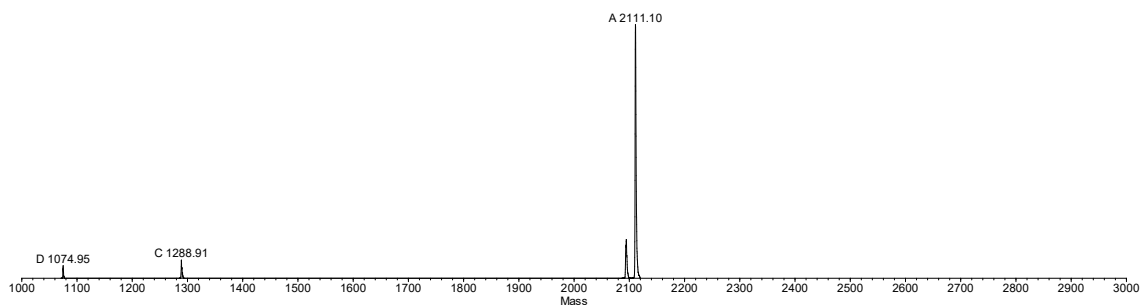


Figure S15 – ESI-MS analysis of purified **GP-4**.

**Peak A:** **GP-4**, Y\*NAAN[Glc(OAc)<sub>4</sub>]VTLN[Glc(OAc)<sub>4</sub>]TTGTL-NH<sub>2</sub>, C<sub>90</sub>H<sub>138</sub>N<sub>18</sub>O<sub>40</sub>, Exact mass calcd.: 2110.93; found: 2111.10.

**GP-5 synthesized on TentaGel R RAM resin**

Sequence: Y\*TVVNATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>

Glc-protected precursor of [Y<sup>1389</sup>,N<sup>1398</sup>(Glc)]HMW1ct(1389-1403)

Resin: TentaGel R RAM (loading 0.18 mmol/g)

Yield of the crude glycosylated peptide **GP-5** before purification: 11.5 mg, 36%.

Yield of the glycosylated peptide **GP-5** after purification: 1 mg, 3%.

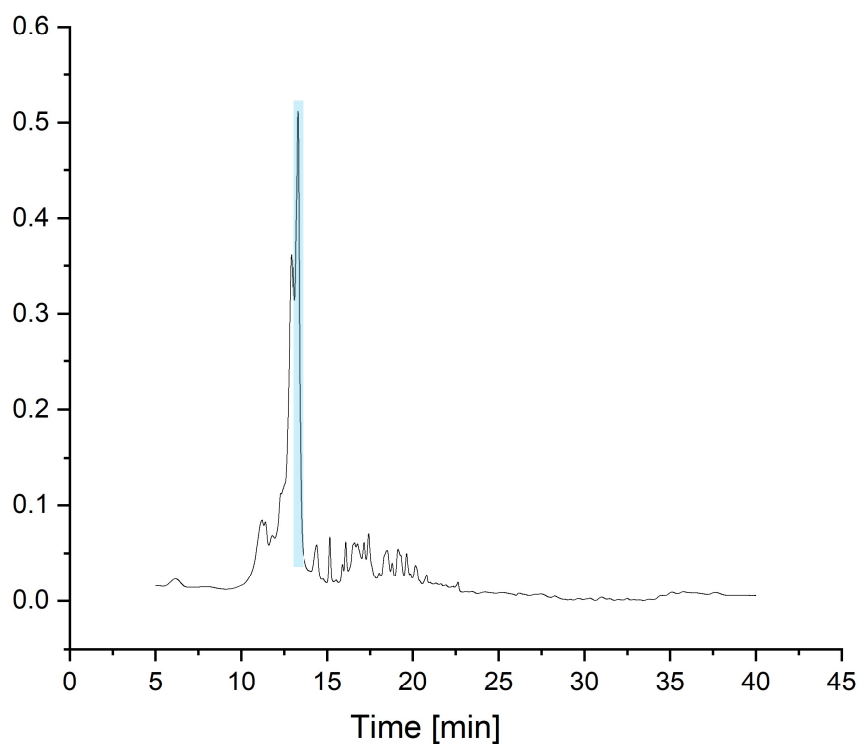


Figure S16 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-5** (peak highlighted in blue) synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

\*Tyr was introduced at the N-terminus to improve UV-HPLC detection.

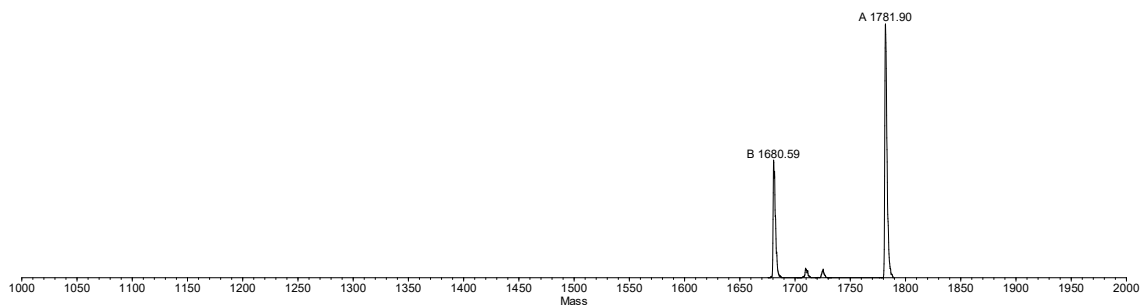


Figure S17 – ESI-MS analysis of **GP-5**.

**Peak A:** **GP-5**, Y\*TVVNATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>, C<sub>74</sub>H<sub>115</sub>N<sub>19</sub>O<sub>32</sub>, Exact mass calcd.: 1781.80; found: 1781.90.

**Peak B:** **Des-Thr-GP-5**, C<sub>70</sub>H<sub>108</sub>N<sub>18</sub>O<sub>30</sub>, Exact mass calcd.: 1680.73; found: 1680.59.

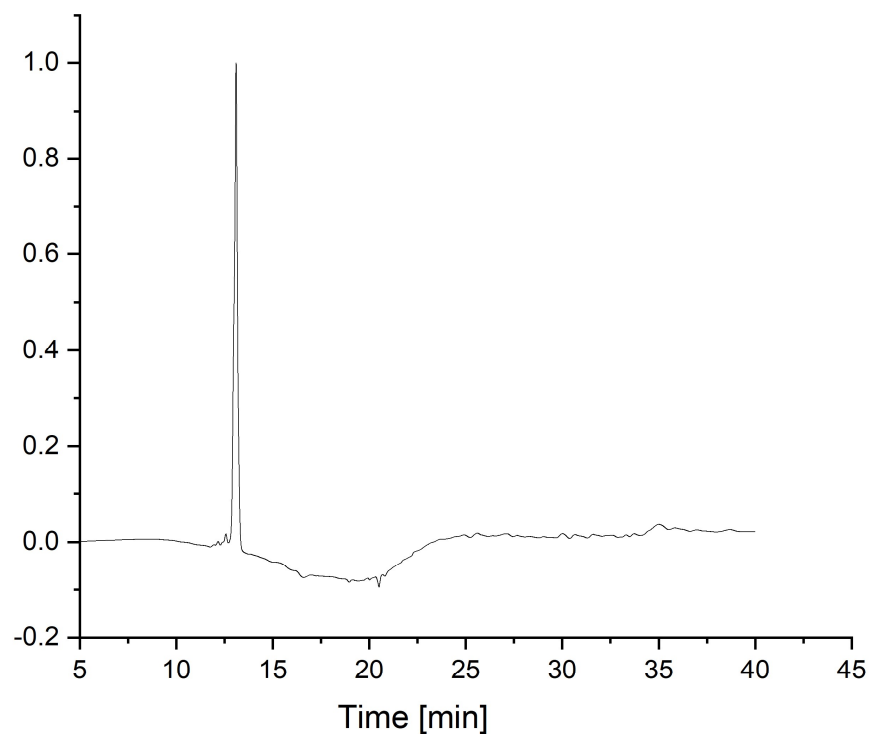


Figure S18 – Analytical HPLC chromatogram (recorded at 220 nm) of purified **GP-5** synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

**GP-6 synthesized on TentaGel R RAM resin**

Sequence: Y\*TVVN[Glc(OAc)<sub>4</sub>]ATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>

Glc-protected precursor of [Y<sup>1389</sup>,N<sup>1393</sup>(Glc),N<sup>1398</sup>(Glc)]HMW1ct(1389-1403)

Resin: TentaGel R RAM (loading 0.18 mmol/g).

Yield of the crude glycosylated peptide **GP-6** before purification: 8 mg, 21%.

Yield of the glycosylated peptide **GP-6** after purification: 1.5 mg, 4%.

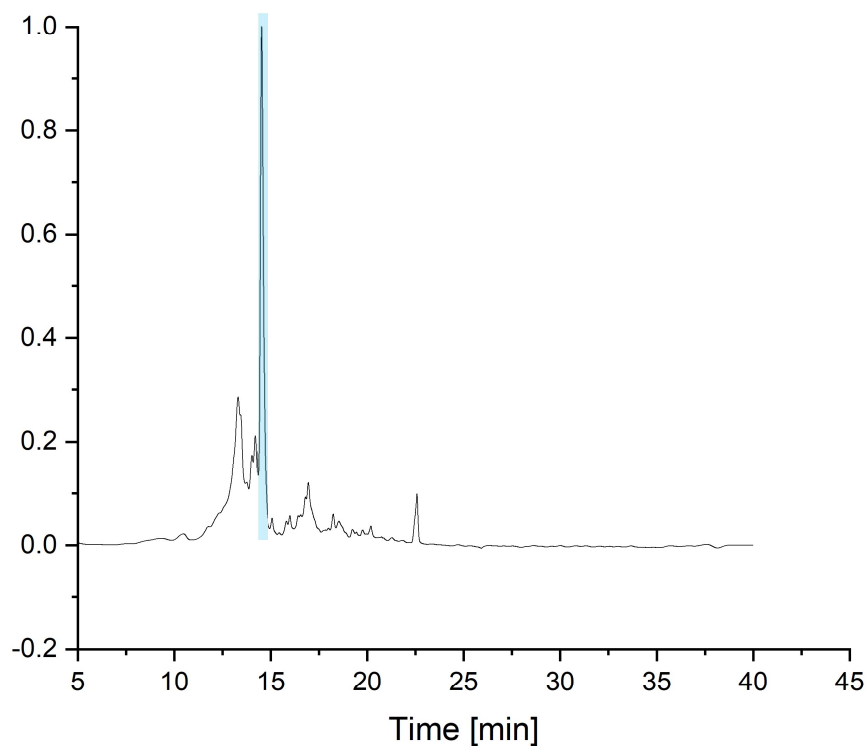


Figure S19 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-6** (peak highlighted in blue) synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

\*Tyr was introduced at the N-terminus to improve UV-HPLC detection.

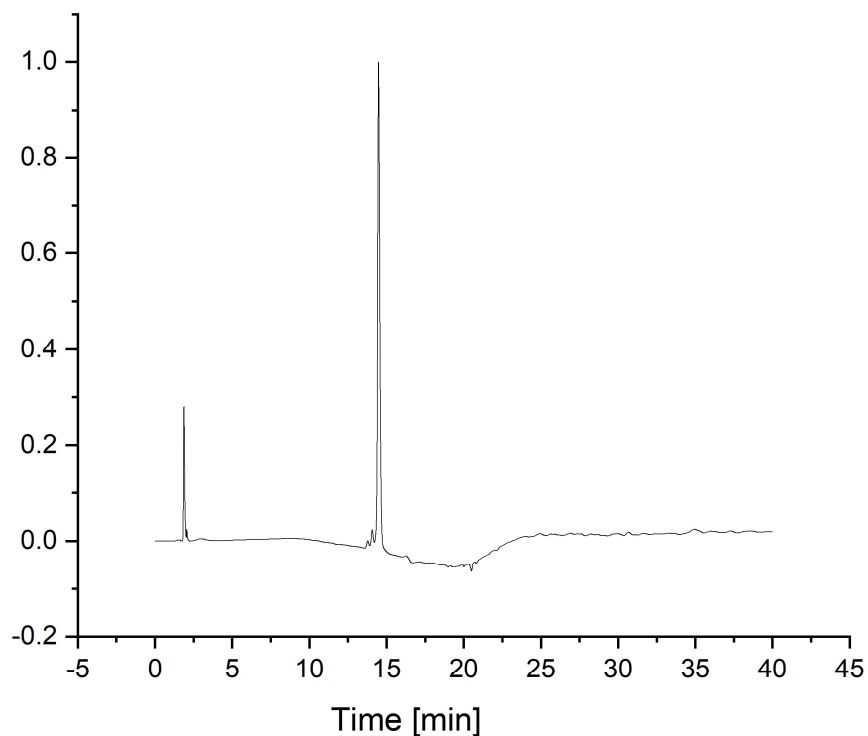


Figure S20 – Analytical HPLC chromatogram (recorded at 220 nm) of purified **GP-6** synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

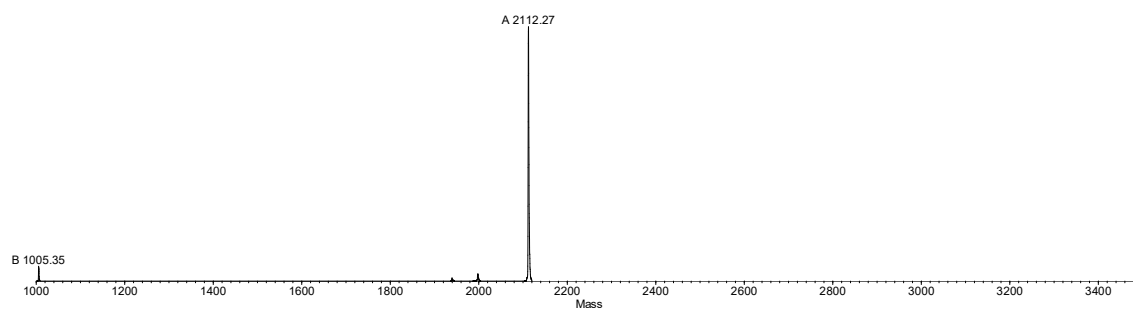


Figure S21 – ESI-MS analysis of purified **GP-6**.

**Peak A:** **GP-6**, Y\*TVVN[Glc(OAc)<sub>4</sub>]ATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>, C<sub>88</sub>H<sub>132</sub>N<sub>19</sub>O<sub>41</sub>, Exact mass calcd.: 2112.10; found: 2112.27.

**GP-7 synthesized on TentaGel R RAM resin**

Sequence: Y\*ALGN[Glc(OAc)<sub>4</sub>]HTVVN[Glc(OAc)<sub>4</sub>]ATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>

Glc-protected precursor of [N<sup>1388</sup>(Glc),N<sup>1393</sup>(Glc),N<sup>1398</sup>(Glc)]HMW1ct(1384-1403)

Resin: TentaGel R RAM (loading 0.18 mmol/g)

Yield of the crude glycosylated peptide **GP-7** before purification: 6.5 mg, 12%.

Yield of the glycosylated peptide **GP-7** after purification: 1 mg, 2%.

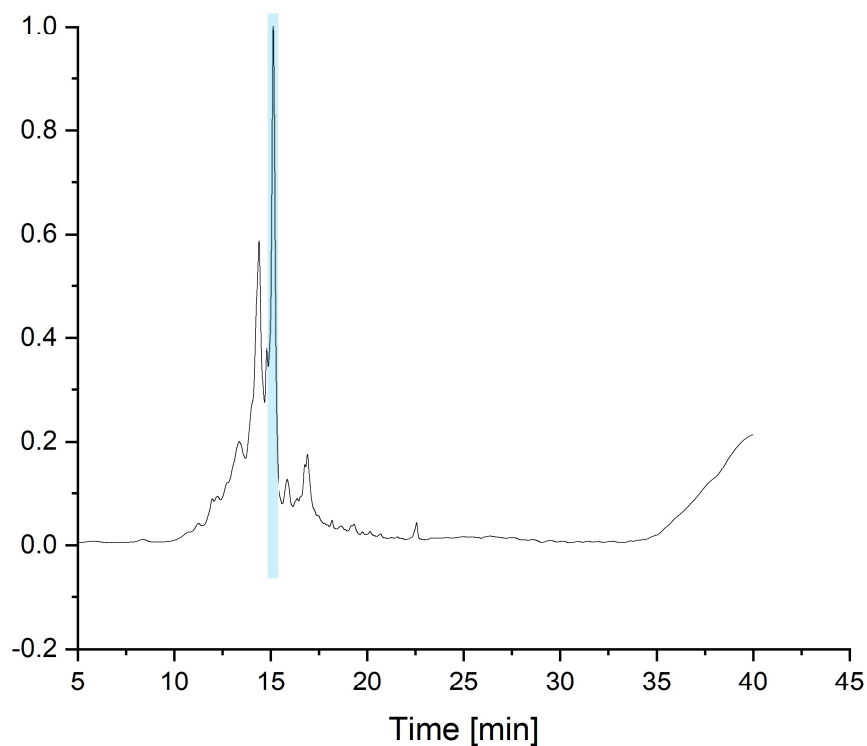


Figure S22 – Analytical HPLC chromatogram (recorded at 220 nm) of crude **GP-7** (peak highlighted in blue) synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).

\*Tyr was introduced at the N-terminus to improve UV-HPLC detection.

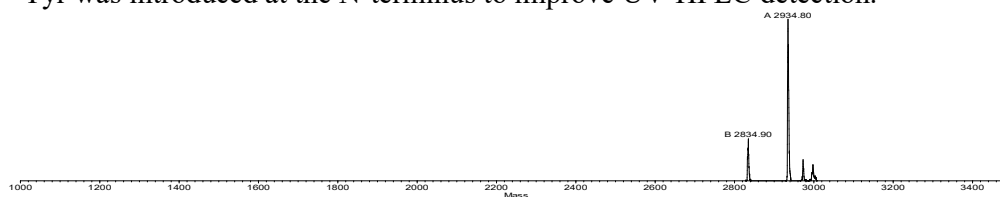


Figure S23 – ESI-MS analysis of **GP-7**.

**Peak A:** **GP-7**, Y\*ALGN[Glc(OAc)<sub>4</sub>]HTVVN[Glc(OAc)<sub>4</sub>]ATNAN[Glc(OAc)<sub>4</sub>]GSGSV-NH<sub>2</sub>, C<sub>123</sub>H<sub>182</sub>N<sub>27</sub>O<sub>56</sub>, Exact mass calcd.: 2934.23; found: 2934.80.

**Peak B:** **Des-Thr-GP-7**, C<sub>119</sub>H<sub>176</sub>N<sub>26</sub>O<sub>54</sub>, Exact mass calcd.: 2833.17; found: 2834.90.

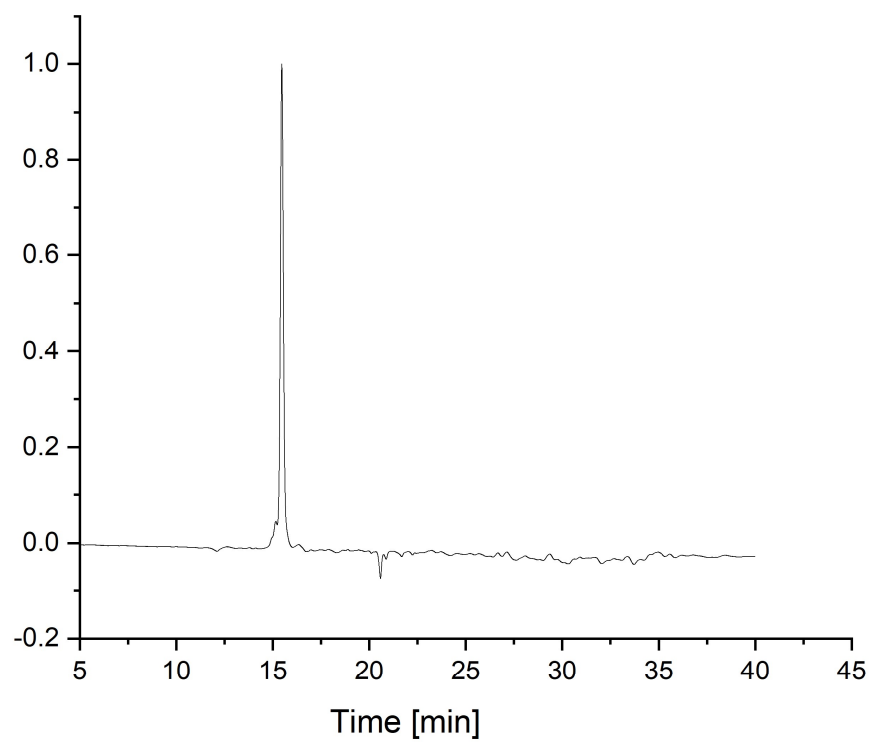


Figure S24 – Analytical HPLC chromatogram (recorded at 220 nm) of purified **GP-7** synthesized on TentaGel R RAM resin (loading 0.18 mmol/g).