Electronic Supplementary Information for the article

A quantitative and site-specific chemoenzymatic glycosylation approach for PEGylated MUC1 peptides

Claudia Bello, Karine Farbiarz, Jan F. Möller, Christian F.W. Becker, Tilo Schwientek

Material and methods

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and Fmoc-His(Trt)-Wang resin were purchased from Novabiochem. *tert*-Butoxycarbonyl (Boc)-protected amino acids and Boc-Leu-PAM resin from Bachem (Bubendorf, Switzerland) and Orpegen (Heidelberg, Germany). Trifluoroacetic acid was purchased from Roth (Karlsruhe, Germany). Fmoc-PEG_{27}-COOH was from Polypure (Norway). Hydrogen fluoride was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich at the highest purity available and used without any further purification.

Product analysis and reaction monitoring by analytical RP-HPLC

Products analysis and reactions monitoring were performed by analytical RP-HPLC on a Beckman - System Gold instrument. Column: C4, 150 x 4.6 mm, 5 µm particle size, Grace Vydac. Flow: 1 ml/min. Gradient: from 5% to 65% buffer B in buffer A over 30 min. Buffer A: 0.1% v/v TFA in ddH_2O, Buffer B: 0.08% v/v TFA in acetonitrile. or on a Dionex Ultimate 3000 instrument (Columns: C4 Kromasil 300-5-C4 150 × 4.6 mm, 5 µm particle size and BioBasic-4 150 × 4.6 mm, 5 µm particle size, Thermo Fisher). Chromatograms are baseline corrected.

Crude peptides were purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a Varian Pro Star system (according to the amount and hydrophobicity of the peptides to be purified, different columns were used: C4 column (250 x 22 mm, 5 µm particle size, Protein C4, Grace Vydac), C4 column (250 x 10 mm, 5 µm particle size, Protein C4, Grace Vydac) and C18 column (250 x 10 mm, 5 µm particle size, Kromasil)) or on a Beckman System Gold instrument, column: Vydac 218TP5415-C18 (4.6 X 15 cm, 5 µm particle diameter, Grace Vydac) equipped with a 218GD54 guard (Grace Vydac). Buffers A and B were used as eluents.

Detection for all the chromatographic methods occurred at 214 nm and 280 nm wavelengths.

ESI and MALDI-TOF mass spectrometry

MALDI mass spectrometry

Reaction monitoring by automatic acquisition of MALDI-TOF MS spectra was performed on a Bruker ultraFleXtreme mass spectrometer. Spectra were recorded in the positive ion mode with external calibration using a pulsed UV laser beam (nitrogen laser, λ = 337 nm) and an acceleration voltage of 20 kV. The reflector voltage was set to 22.5 kV. Methods for automatic acquisition were defined in the autoexecute module of the FlexControl software (Bruker Daltonics).

ESI mass spectrometry

ESI-MS analyses were performed on a PTM Discovery System (HCTultra ETD II, Bruker) coupled with an online nano-LC system (Ultimate, LC-Packings) and a PepMap C18 column (75 µm ID, 5 cm). Ions were scanned with 8100 amu/sec in a range of m/z 300 – 2500 in MS mode and m/z 100 – 3000 in MS/MS mode. MS/MS spectra were generated by CID (helium) or ETD fragmentation. Static and data-dependent acquisition of spectra was controlled by the Compass software.

The peptide mass spectra for final analysis were determined either by positive ion mode electrospray ionization (ESI) using a Finnigan LCQ-classic mass spectrometer and a Waters AutoPurification HPLC/MS System equipped with a Waters 3100 Mass detector, or by Matrix Assisted Laser Desorption/Ionization (MALDI) using a Voyager-DE Pro MALDI (Applied Biosystems) in positive ion mode.

Solid phase peptide synthesis

Mucin peptides **A18**, **TA18** and **TCA18** were synthesized manually on a solid support using the fluorenylmethoxycarbonyl (Fmoc) strategy[1]. Preloaded Fmoc-Alanine-Wang-polystyrene resin (Fmoc-Ala-Wang-PS) was used as the solid support for the synthesis of the peptides on a 0.2 mmol scale. N-terminal Fmoc deprotection was performed by incubating the resin twice with a piperidine solution (20% v/v in DMF) for 3 and 7 min, respectively. After washing with DMF, the resin was incubated for 30 min with the preactivated amino acid (2.5 eq, activated with 2.4 eq HBTU (0.5M, DMF) and 5.0 eq DIEA, 3 min) and washed again with DMF before starting a new deprotection step. Coupling and deprotection reactions were monitored by ninhydrine test.[2] The amino acids used in the synthesis were carrying orthogonal side-chain protecting groups as follow: Thr(O^tBu), Ser(O^tBu), Arg(Pbf), Asp(O^tBu), His(Trt), Cys(Trt). To improve synthesis yields, the pseudoproline dipeptides Gly-Ser($\psi^{Me,Me}$Pro)-OH and Val-Thr($\psi^{Me,Me}$Pro)-OH were used.[3, 4] After coupling of the last amino acid, the resin was washed with DMF, DCM and MeOH and dried under vacuum. The solid support was then removed by incubating the peptidyl-resin with a mixture of TFA/TIS/H_2O (92.5:5:2.5) for 3h at room temperature. The free peptide was precipitated by addition of cold diethyl ether and centrifugation. Finally, the supernatant was removed and the peptide washed with cold diethyl ether, dissolved in a mixture of H_2O/CH_3CN 1:1 and then freeze-dried.

Crude peptides were purified by semi-preparative or preparative RP-HPLC (according to the amount and hydrophobicity of the peptides to be purified, different columns were used: C4 column (250 x 22 mm, 5 µm particle size, Protein C4, Grace Vydac), C4 column (250 x 10 mm, 5 µm particle size, Protein C4, Grace Vydac) and C18 column (250 x 10 mm, 5µm particle size, Kromasil). Flow: 3 mL/min (semi-preparative column) and 10 mL/min (preparative column). Gradients: linear, from 5% to 65% buffer B in Buffer A, linear from 35% to 65% buffer B in buffer A). Yields were calculated by peak integration of analytical RP-HPLC profiles.

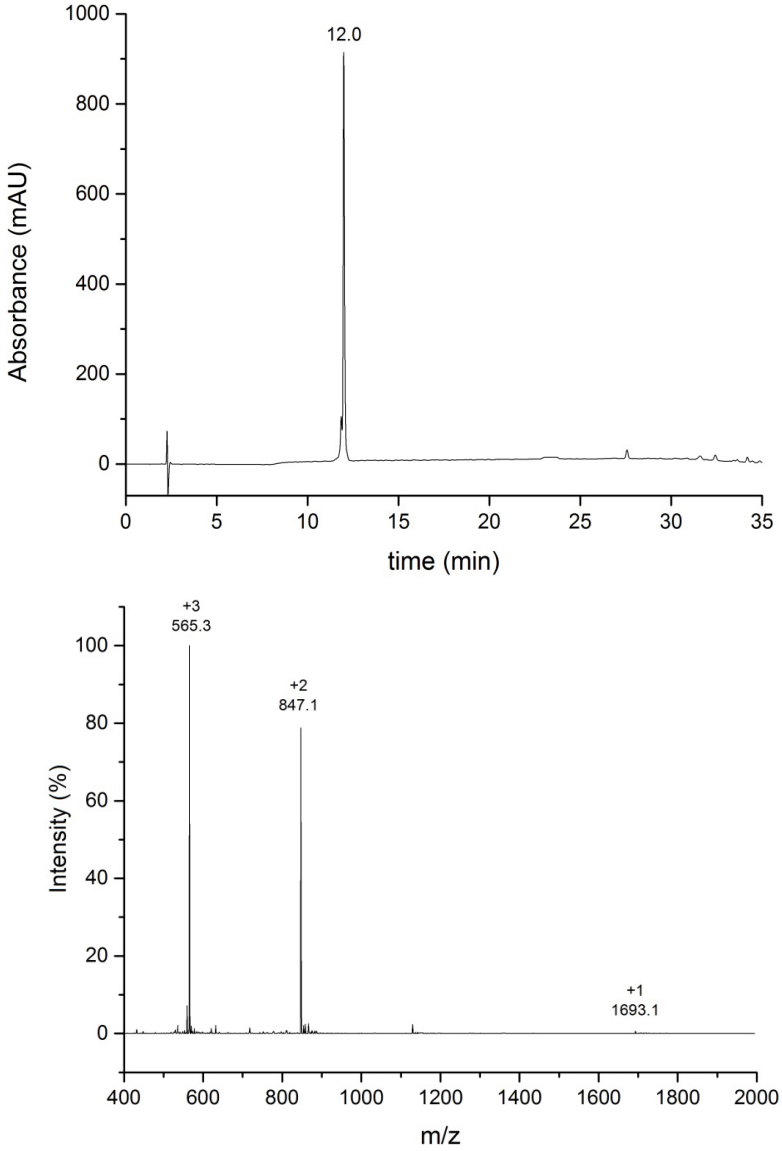
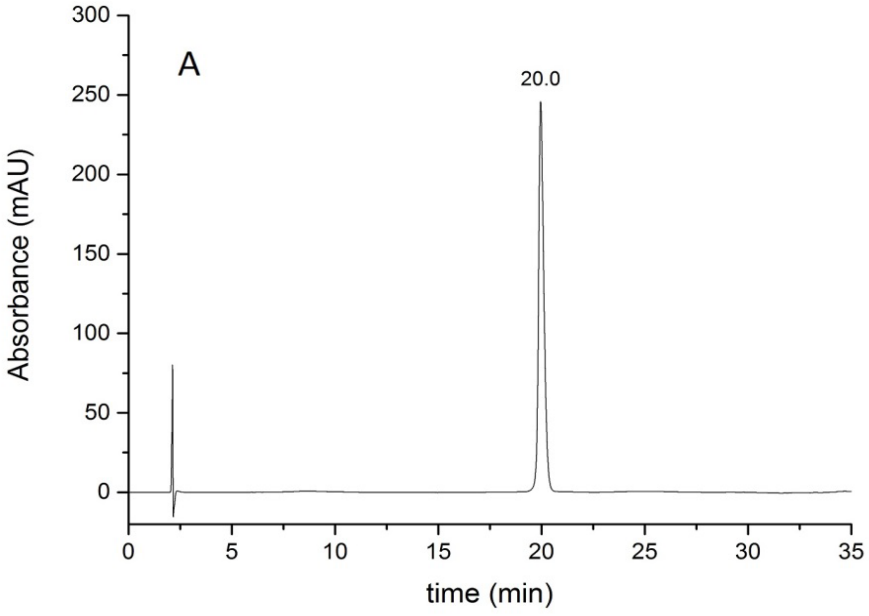
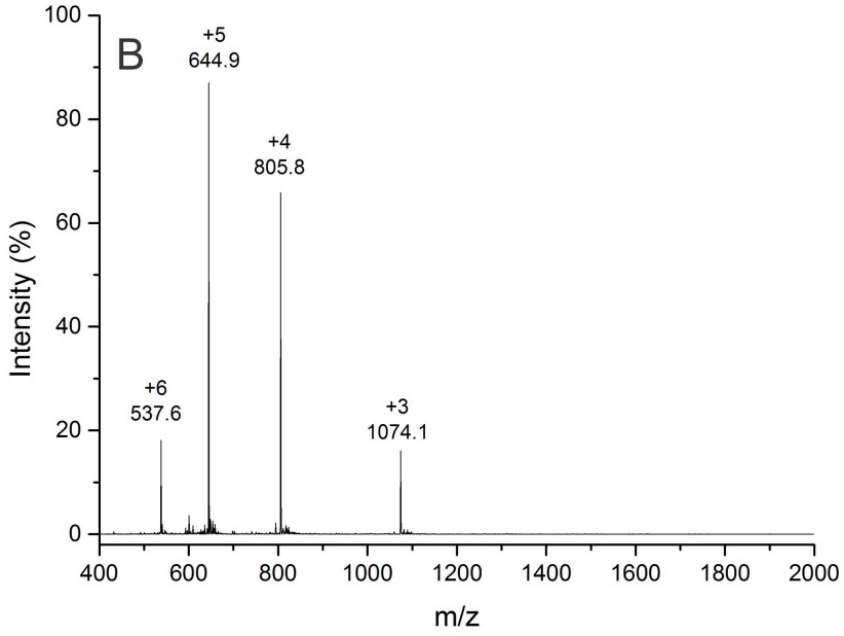


Figure S1: RP-HPLC (C4) profile (*A*) and ESI-MS analysis (*B*) of purified peptide A18. ESI-MS data were detected by direct injection of the peptide eluting at 12.0 min. Calculated mass for $C_{70}H_{113}N_{23}O_{26}$: 1691.8 Da, found: 1693.1 Da ($[M+H]^{+}$), 847.1 Da ($[M+2H]^{2+}$) and 565.3 ($[M+3H]^{3+}$). A18 was analysed in order to ensure sufficient quality for subsequent PEGylation. For overall yield please see figure S2.

Peptide conjugation with monodisperse PEG

PEGylation of the mucin peptides **A18**, **TA18** and **TCA18** was performed as follows: the peptidyl-resin was swollen in DMF for 2h. Then, the N-terminal Fmoc protecting group was removed by incubation with piperidine (20% v/v in DMF) for 3 min and once again for 7 min. After extensive washing in DMF, a solution of monodisperse Fmoc-PEG_{27}-OH (1.38 eq, preactivated for 3 min with HBTU (0.95 eq, 0.5M in DMF) and DIEA (2eq)) was added and the suspension was incubated at room temperature for 2h. The supernatant was removed and the peptidyl resin was washed with DMF, DCM and MeOH. Then, the peptide was removed from the solid support using the same method as for non-PEGylated **A18**, **TA18** and **TCA18** peptides (see above) and purified by preparative RP-HPLC using a C4 column (flow 10 ml/min, gradient from 35% to 65% buffer B in buffer A).





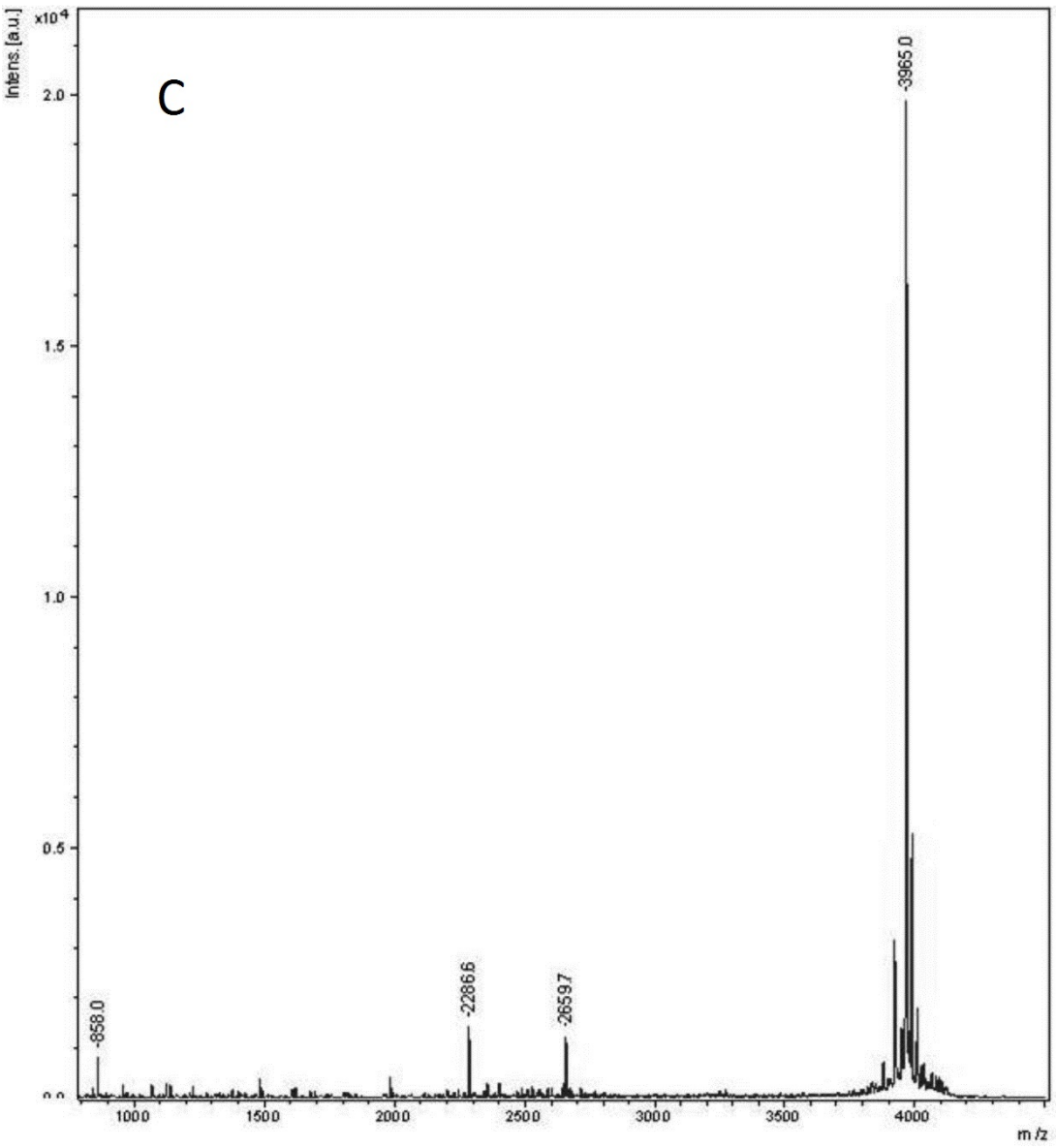


Figure S2: RP-HPLC (C4) profile (*A*) and ESI-MS analysis (*B*) of the PEGylated peptide PA18 ESI-MS data were recorded by direct injection of the peptide eluting at 20.0 min. Calculated mass for $C_{144}H_{240}N_{24}O_{57}$ is 3218.6 Da, found 1074.1 Da ($[M+3H]^{3+}$), 805.8 Da ($[M+4H]^{4+}$), 644.9 Da ($[M+5H]^{5+}$) and 537.6 Da ($[M+6H]^{6+}$). The overall yield of peptide synthesis, including PEGylation and purification, was 86% based on the synthesis scale. **(C) MALDI-MS analysis of PEGylated PTA18**. Calculated Mass for $C_{173}H_{289}N_{34}O_{70}$: 3964.3 Da, found 3965.0 Da ($[M+H]^{+}$).

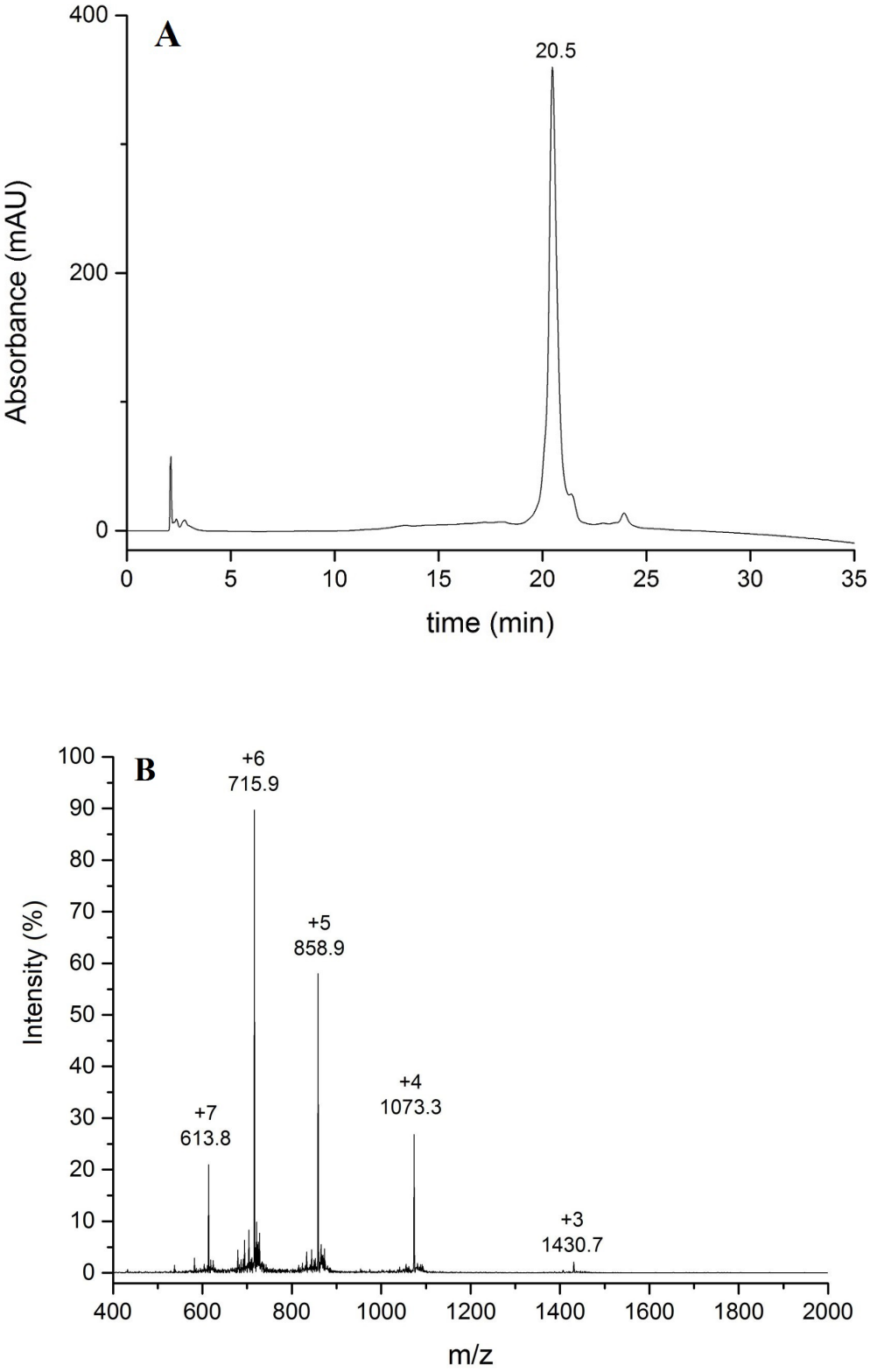


Figure S3: **RP-HPLC (C4) profile (*A*) and ESI-MS analysis (*B*) of the PEGylated peptide PTCA18.** ESI-MS data were recorded by direct injection of the peptide eluting at 20.5 min. Calculated mass for $C_{191}H_{304}N_{35}O_{73}S$ is 4288.0 Da, found: 1430.7 Da ($[M+3H]^{3+}$), 1073.3 ($[M+4H]^{4+}$), 858.9 ($[M+5H]^{5+}$), 715.9 ($[M+6H]^{6+}$), 613.8 ($[M+7H]^{7+}$). The final yield of the purified PEGylated peptide was 20% based on the synthesis scale.

Dithiomethane modification of peptide cysteines

Peptide sulfhydryl group modification of **PTCA18** with methylmethanethiosulfonate was performed in 10 mM PBS, pH 7.5. At scales ranging from 100 µg to 5 mg, agitated incubation of the unmodified peptide with a 100-fold molar excess of methylmethanethiosulfonate for 3h at RT resulted in full conversion to the dithiomethylated product **PTCA18*** with a mass increment of 46 Da.[5] Quantitative conversion into the cysteine-protected derivative was confirmed by the absence of the **PTCA18** educt peak in MALDI-TOF MS analyses of the crude reaction material. **PTCA18*** was purified by solid phase extraction or preparative RP-HPLC prior to further use.

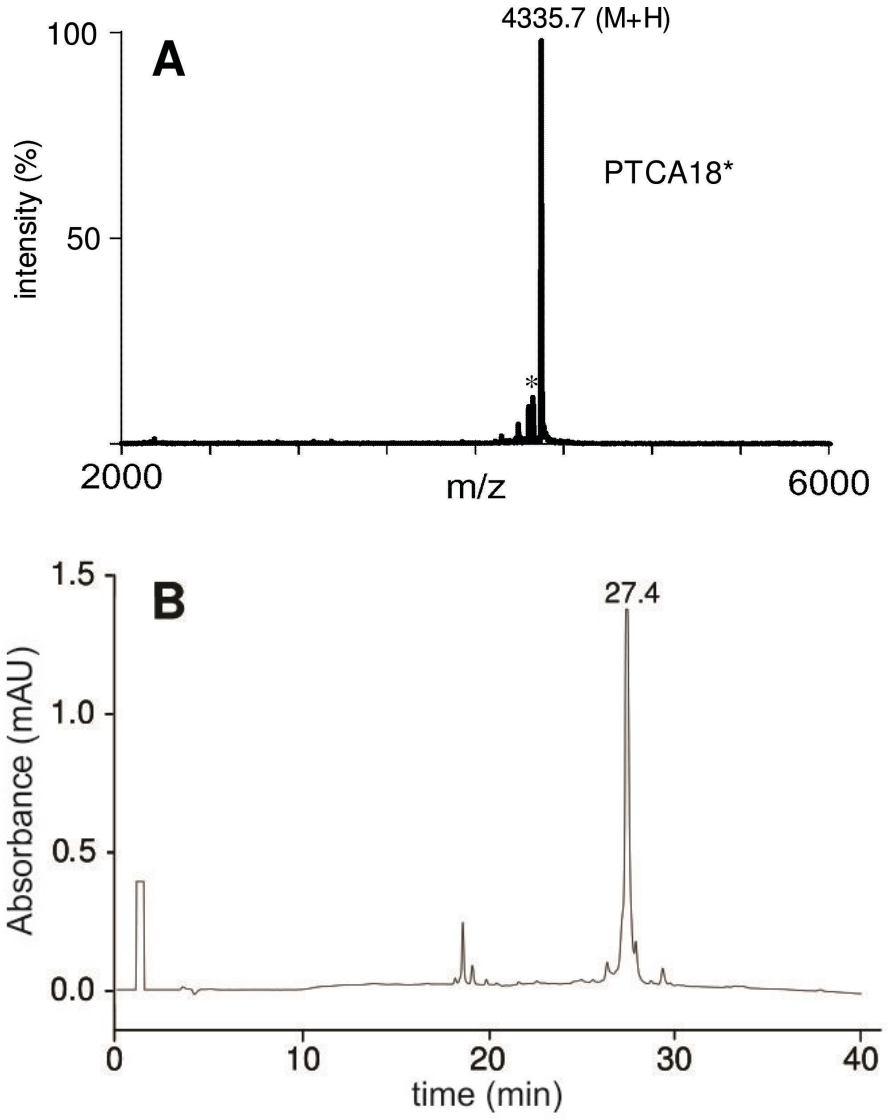


Figure S4: **Quantitative thiomethylation of PTCA18.** (***A***), MALDI-TOF MS analysis of crude **PTCA18*.** The calculated mass for $C_{192}H_{307}N_{35}O_{73}S_2$ is 4336.0 Da, found 4335.7 Da. Minute traces of **PTCA18** at m/z 4290.0 (Δm/z -46) are indicated by the *asterisk*. Monoisotopic masses of positive ions $[M+H]^+$ are displayed. (***B***), RP-HPLC (C18) chromatogram of 20 µg crude MMTS-treated peptide. **PTCA18*** elutes at 27.4 min (75 % eluent B).

Synthesis of peptide thioesters

The MUC1 thioester peptide was synthesized via Boc-based Solid Phase Peptide Synthesis starting from a styrene-divinylbenzene resin carrying a PAM linker and a thioester-generating linker. The first amino acid was coupled manually: 10 eq were preactivated with HBTU (9 eq, 0.5 M in DMF) and DIEA (30 eq) for 1 minute and then added to the resin. The suspension was incubated at room temperature for 15 min with occasional stirring. Then, the supernatant was removed and the coupling procedure repeated by incubating the resin with a fresh solution of activated amino acid for 15 min. Subsequently, the resin was washed with DMF, DCM and MeOH and dried under vacuum. The efficiency of the coupling was checked by ninhydrine test. Then, the dry resin was transferred into the reaction vessel of the Boc-synthesizer (ABI 433A Peptide Synthesizer from Applied Biosystems), swollen with DMF for 1h and further peptide synthesis steps continued automatically. Amino acids with side chains protected with acid resistant protecting groups were used in the synthesis. The peptide was released from the solid support with anhydrous HF using *p*-cresol as scavenger. After removal of HF under vacuum, the peptide was precipitated with cold diethyl ether and filtered. The precipitated peptide and the resin were washed twice with cold diethyl ether and the peptide dissolved in H_2O/CH_3CN 1:1, separated from the resin beads and freeze-dried. Finally, the purified peptide was obtained after semi-preparative RP-HPLC (C4 column, 3 ml/min flow rate, gradient from 25% buffer B in buffer A over 60 min) in 3% yield.

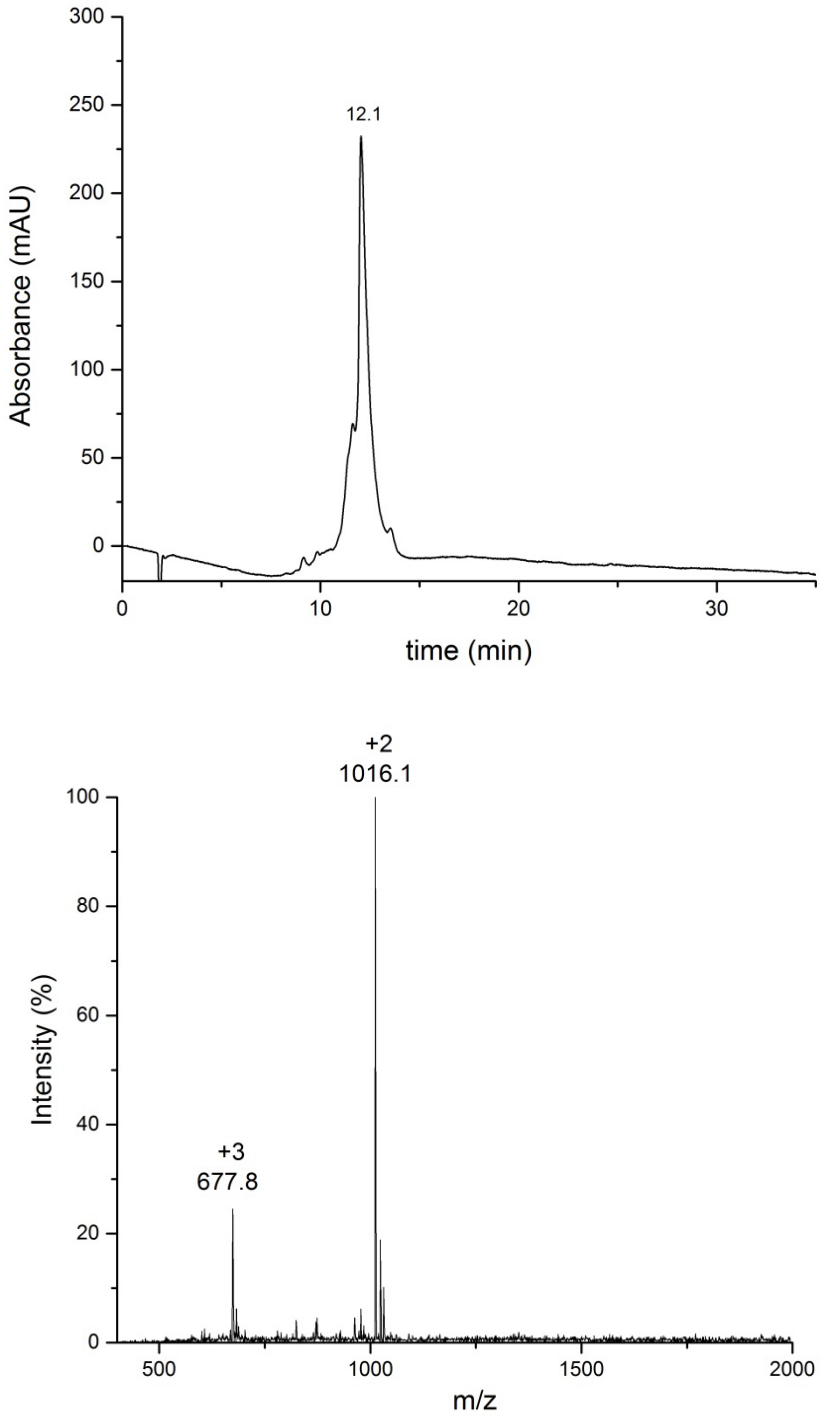


Figure S5: **RP-HPLC (C4) profile (*A*) and ESI-MS analysis (*B*) of the MUC1 peptide α-thioester A20-SR.** ESI-MS data were recorded by direct injection of the peptide eluting at 12.1 min**.** Calculated mass for $C_{144}H_{240}N_{24}O_{57}$: 2031.0 Da, found: 1016.1 Da ($[M+2H]^{2+}$), 677.8 Da ($[M+3H]^{3+}$). The overall yield of the **A20-SR** peptide was 3% based on the synthesis scale.

Enzymatic *in vitro* glycosylation

All peptides were site-specifically glycosylated *in vitro* using purified recombinant *Drosophila* glycosyltransferases. *DGalNAcT1* was expressed as secreted N-terminally truncated protein and purified as previously reported.[6,7] The cloning, expression and purification of glycosyltransferases *dC1GalT1* and *dGlcAT-BSII* will be described elsewhere.

Peptide *in vitro* glycosylation with *Drosophila* polypeptidyl: N-acetyl-D-galactosaminyltransferase 1

For the initial addition of *N*-acetyl-D-galactosamine (i.e. of the Tn-antigen) to the peptides, the polypeptidyl GalNAc-transferase *dGalNAcT1* was used. Glycosylation of the peptide conjugates was performed by dissolving 50 to 200 µg of the peptide (1 mg/ml) in a solution of UDP-GalNAc (2 mM), Triton X-100 (0.1% v/v) and $MnCl_2$ (10 mM) in Tris-HCl buffer (25 mM, pH 7.4). Then the glycosyltransferase was added and the reaction mixture was shaken for 18 h at 37°C.[7] The progress of the *O*-glycosylation reaction was monitored by MALDI-TOF MS analyses in negative ion mode as described above. The mass spectrometric analyses revealed highly efficient formation of the Tn-glycopeptide conjugates (detected by a mass increment of 203.1 Da for 1 HexNAc). The enzymatic reaction endpoint was reached after overnight incubation and resulted into quantitative conversion as confirmed by the total disappearance of the unglycosylated educt mass.

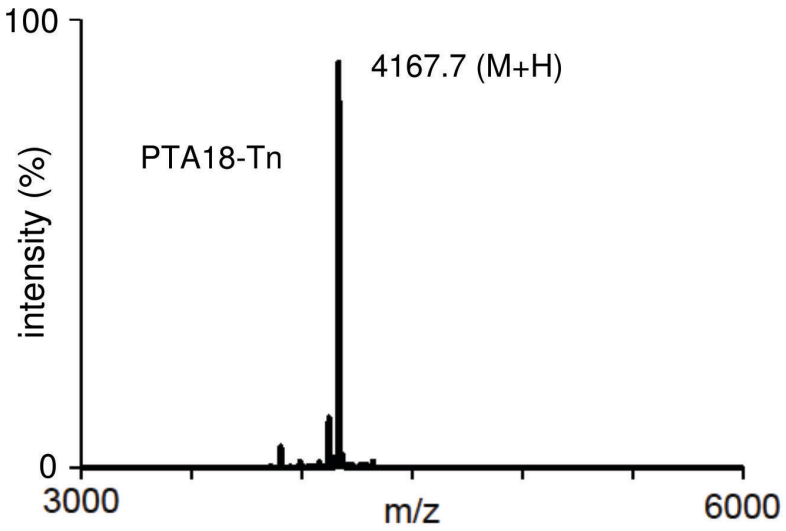


Figure S6: MALDI-MS reaction monitoring of the *dGalNAcT1* glycosylation reaction towards PTA18-Tn. 100 µg of chemically synthesized **PTA18** were glycosylated with purified *dGalNAcT1*. The calculated mass for $C_{181}H_{302}N_{35}O_{75}$ is 4167.4 Da, found 4167.7 Da ($[M+H]^+$). No trace of the **PTA18** educt mass at 3964.3 (Δm/z -203.1) is detectable.

Enzymatic glycosylation with *Drosophila* core 1 β1-3-galactosyltransferase 1

Glycopeptides carrying the Tn-antigen were further glycosylated *in vitro* using purified recombinant *Drosophila dC1GalT1*. This glycosyltransferase is able to transfer galactose in β1-3 configuration to the initial GalNAc in order to synthesize the T-antigen Galβ1-3GalNAcα-R.[8] Glycosylation reactions with 20 to 200 µg of the Tn-peptide conjugates (1 mg/mL) were performed in a reaction mixture containing 100 mM MES buffer (pH 6.5), 20 mM $MnCl_2$, 0.1% Triton X-100, 20 mM dithiothreitol and 2 mM UDP-Gal. Purified *dC1GalT1* was added and the reaction mixture incubated for 18h at 37°C that quantitatively converted the Tn-antigen into the T-antigen. The completed enzyme reactions resulted in a peptide mass increment of 162.1 Da (for 1 Hexose) with no traces of the educt mass remaining. The O-glycopeptide products were then isolated by micro-GPC and peptide precipitation as described below.

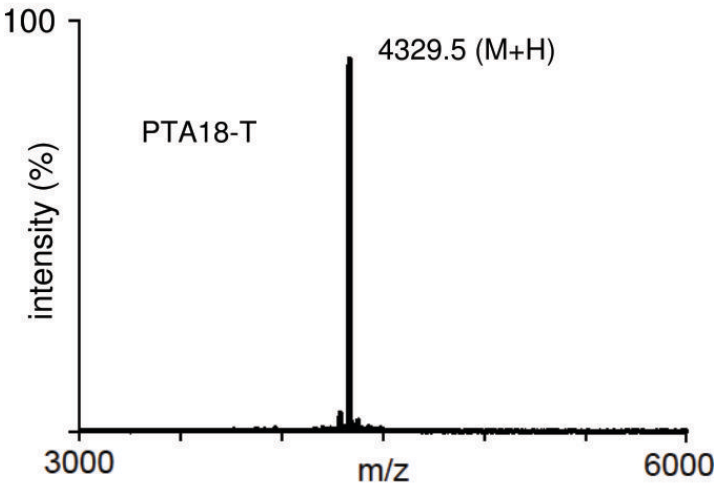


Figure S7: Quantitative enzymatic synthesis of the O-glycopeptide PTA18-T. MALDI-MS spectrum of the completed glycosylation reaction of purified *dC1GalT1* with 50 µg of **PTA18-Tn**. The calculated mass of **PTA18-T is** 4328.1 Da, found 4329.5 Da ($[M+H]^+$). No **PTA18-Tn** educt mass is detectable at 4167.4 Da. Monoisotopic masses of positive ions $[M+H]^+$ are displayed.

In vitro glycosylation with the *Drosophila* β1-3 glucuronyltransferase *dGlcAT-BSII*

Elongation of the peptide-bound sugar chain to the glucuronyl-T antigen (GlcAβ1-3Galβ1-3GalNAcα-R) was carried out *in vitro* using purified recombinant *Drosophila dGlcAT-BSII.* Glucuronylation of 20 μg up to 200 μg of the T-antigen glycosylated peptide conjugates (1 mg peptide/mL) was performed in a reaction mixture containing 100 mM MES buffer (pH 6.5), 10 mM $MnCl_2$, and 2 mM UDP-GlcA. Then the purified β1-3glucuronyltransferase was added and the reaction mixture incubated overnight at 37°C. Conversion of the T-antigen O-glycan into the glucuronyl-T-antigen was followed by MALDI-TOF MS. The analyses confirmed highly efficient β1-3glucuronylation by a peptide mass increment of 176.1 Da. The enzymatic reaction reached full conversion after overnight incubation that was confirmed by the non-detectable educt mass.

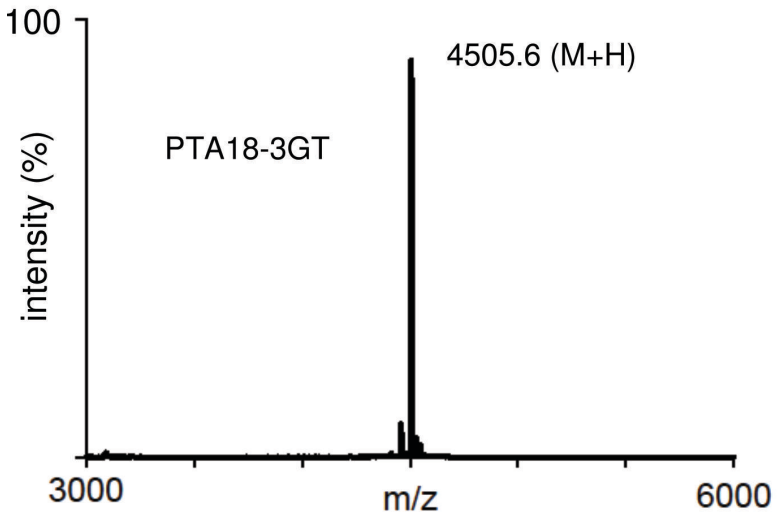


Figure S8: **Enzymatic synthesis of the O-glycopeptide PTA18-3GT.** MALDI-MS reaction monitoring (t = 18h) of the crude enzymatic reaction of *dGlcAT-BSII* with **PTA18-T.** The calculated mass of **PTA18-3GT** is 4505.6 Da, found 4505.6 Da ($[M+H]^+$). The substrate peptide **PTA18-T (**4329.5 Da) is not detectable in the mass spectrum. Monoisotopic masses of positive ions $[M+H]^+$ are displayed.

Glycosylation reaction product separation

The glycosylated peptide conjugates were separated from low molecular weight reaction components by gel permeation chromatography (GPC) on cross-linked dextran spin columns. Micro Bio-Spin columns (Bio-Rad) were prepacked with 0.5 ml of reconstituted Sephadex G-10 (GE LifeSciences) and washed with 5 (5 × 1) column volumes of MQ water. The glycosylation reaction mixture was applied to the top of the GPC gel bed and a stacker volume of water was added to a total volume of 100 µl. The glycosylated peptide conjugates were recovered from the column by centrifugation (500 ×g, 5 min, the recovery efficiency from GPC was higher than 90% (see Fig. S9)) and half of the sample (50 µl) was added to nine sample volumes of ethanol in Eppendorf tubes. The solution was mixed vigorously and the PEGylated peptide precipitated by the addition of two volumes (1 ml) of diethyl ether and 4h incubation at -80°C. The glycopeptide precipitate was harvested by centrifugation at 15000 ×g for 30 min, dried for 30 min at RT and reconstituted in the appropriate buffer prior to the next glycosylation step.

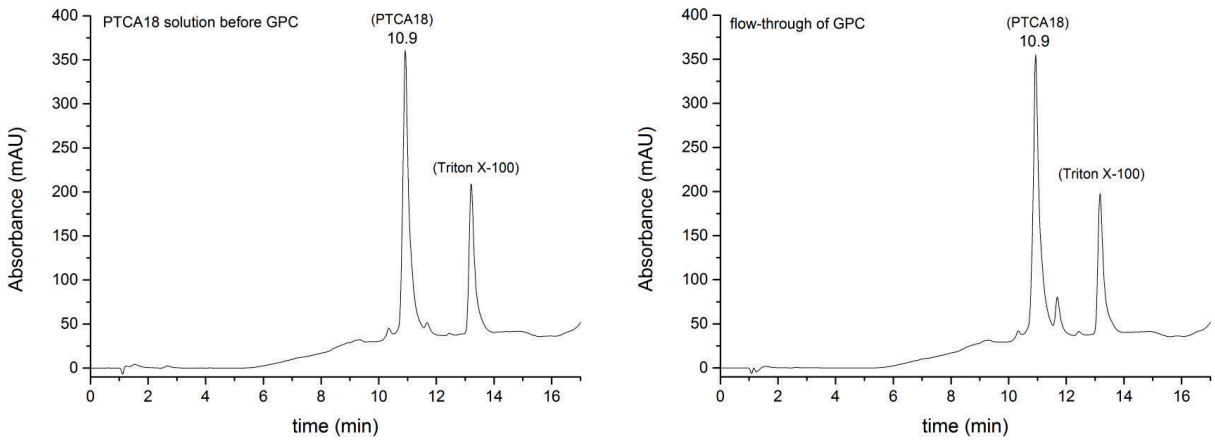


Figure S9. Spin column-Gel Permeation Chromatography of peptide PTCA18. *Left:* HPLC analysis (8 µl injection volume) of a solution of peptide PTCA18 (1mg/ ml), Triton X-100 (0.1% v/v) and $MnCl_2$ (10 mM) in Tris-HCl buffer (25 mM, pH 7.4). *Right:* HPLC analysis (15 µl injection volume) of the flow-through obtained after GPC (70 µl of the PTCA18 solution were applied to the top of the GPC gel bed. Total volume of flow-through recovered: 135 µl) More than 90% of the peptide was recovered.

Peptide release from the polymer and glycopeptide purification

Glycosylated peptide conjugates were isolated from the last glycosylation reaction by micro GPC and precipitation as described above. This was followed by digestion with Tobacco etch virus (TEV) protease resulting in the release of the glycopeptide products from the PEGylated precursors. The reaction was performed in 50 mM Tris-HCl buffer (pH 8.0), 2 mM DTT or 5 mM MESNa, and 1 mM EDTA for 3 h up to overnight incubation at RT. The TEV protease was used in a ratio of 1:50 (w/w) up to 1:20 depending on the peptide substrate. The ongoing reaction was monitored by MALDI-MS until completion. The

released glycopeptides were then separated from PEG_{27}-GDENLYFQ and purified by RP-HPLC as described above.

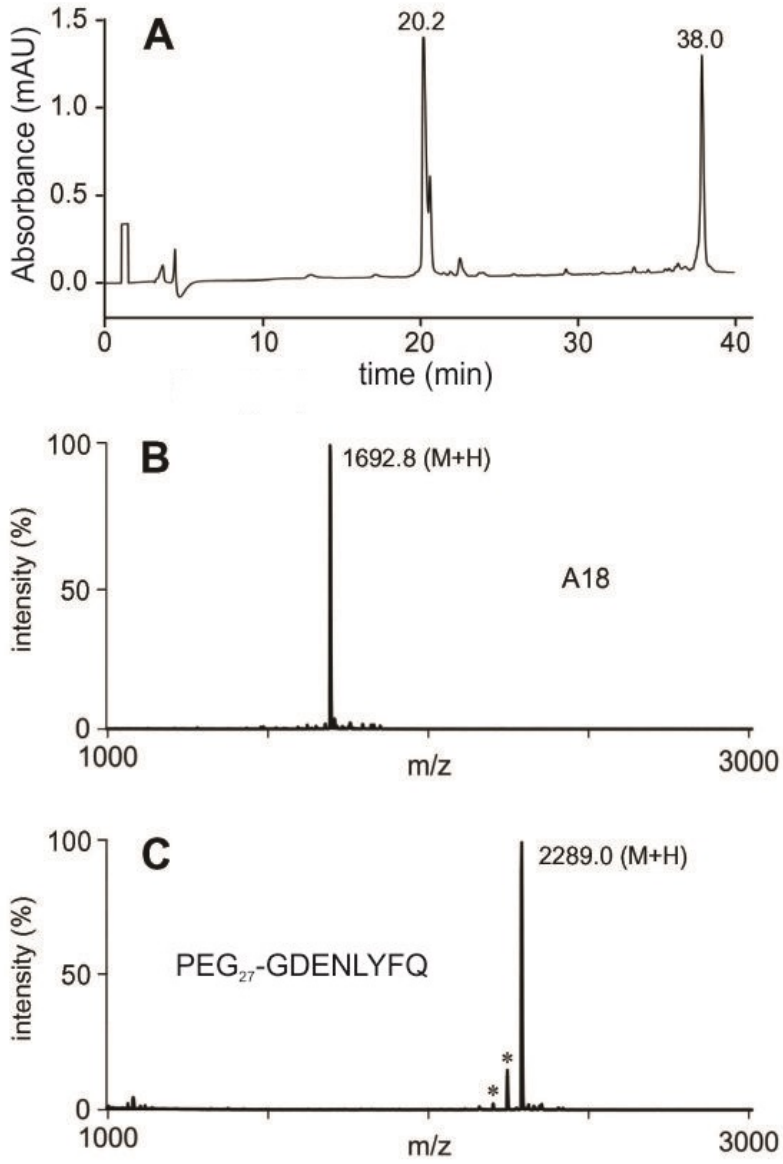


Figure S10. Quantitative release of the A18 peptide by TEV protease digest of PTA18. (***A***) RP-HPLC (C18) profile of 75 µg **PTA18** digested with 1.5 µg Tobacco etch virus protease. The reaction is quantitative based on the absence of undigested starting material. (***B***) MALDI-TOF MS spectrum of the released **A18** eluting at 20.2 min (22 % Eluent B). Calculated mass for $C_{70}H_{113}N_{23}O_{26}$: 1691.8 Da, found 1692.8 Da. (***C***) MS spectrum of the N-terminal Fmoc-PEG-TEV fragment **PEG_{27}-GDENLYFQ** eluting at 38.0 min (100 % eluent B). Calculated mass for $C_{103}H_{177}N_{11}O_{45}$: 2288.2 Da, found 2289.0 Da. Monoisotopic masses of positive ions $[M+H]^+$ are displayed. Minute amounts of PEG_{26}- GDENLYFQ (Δm/z -44) and PEG_{25}- GDENLYFQ (Δm/z -88) that originate from impurities of the PEG starting material are indicated by *asterisks*.

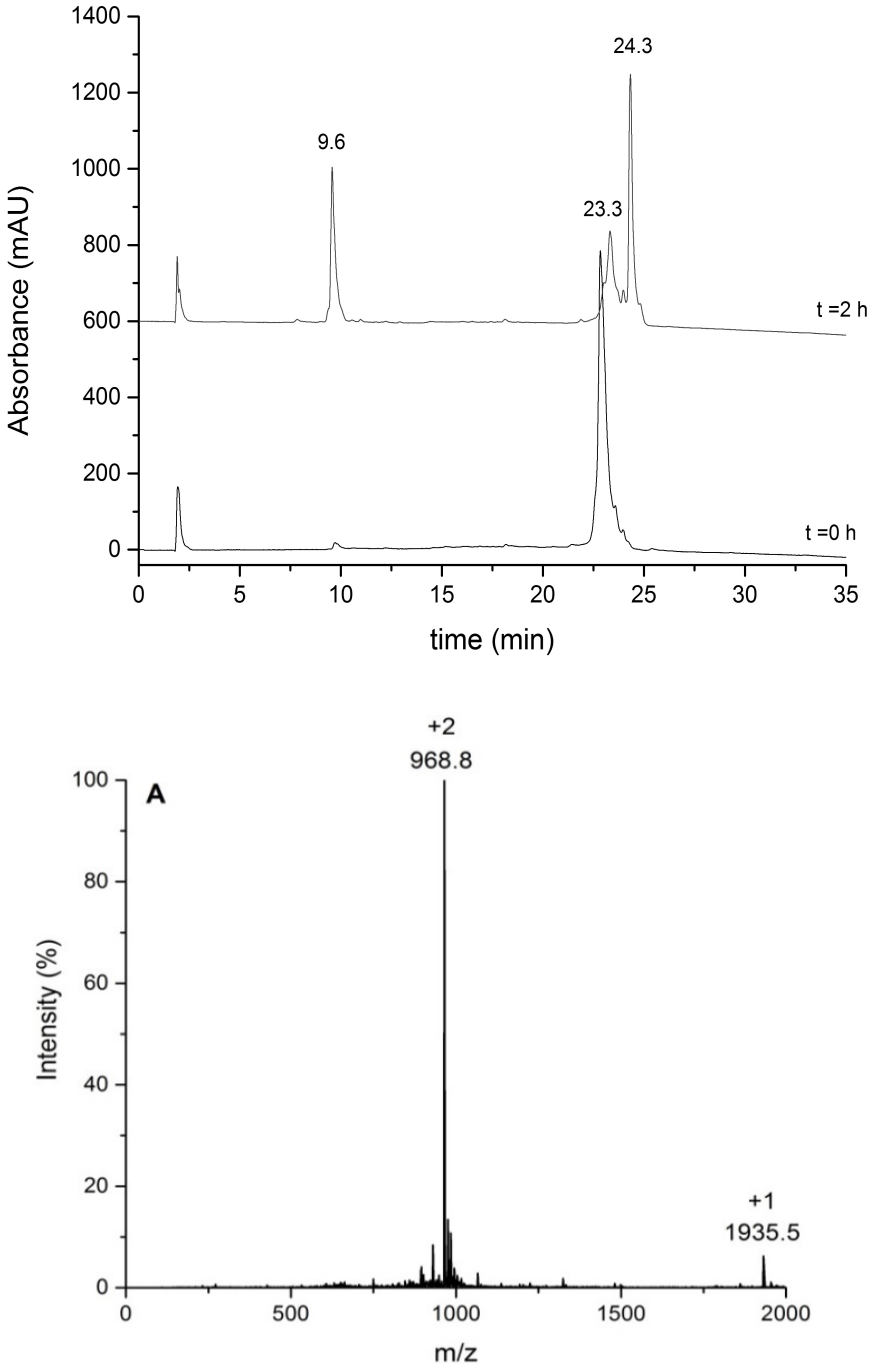


Figure S11. Release of the CA18 peptide by TEV protease digest of PTCA18. (***A***) rpHPLC (C4) profile of **PTCA18** analyzed at start and after 2h of digestion with TEV protease. The peak at 23.3 min represents the undigested starting material, while the peak at 24.3 min contains the N-terminal Fmoc-PEG-TEV fragment **PEG_{27}-GDENLYFQ**. The reaction proceeded to 70% conversion based on LC peak area integration. (***B***) ESI-MS of the released **CA18** peptide eluting at 9.6 min. Calculated mass for $C_{73}H_{118}N_{24}O_{27}S$: 1794.8 Da, found: 1935.5 Da [(M-MESNa)+H]+ (disulfide with MESNa).

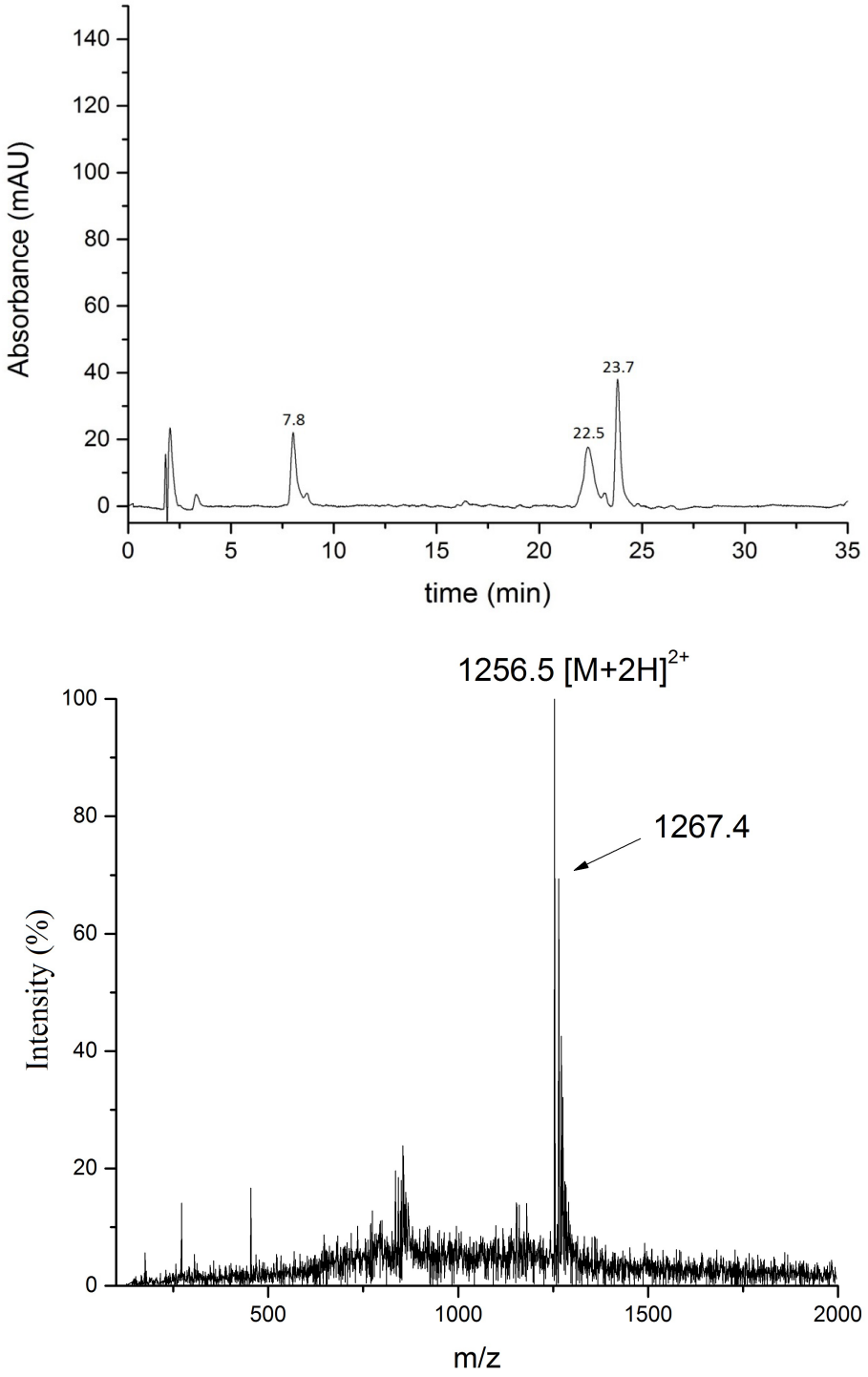


Figure S12. Enzymatic release of CA18-Tn from PTCA18-Tn. (***A***) RP-HPLC chromatogram of the TEV digestion of peptide **PTCA18-Tn** (baseline corrected). The peak at 7.8 min represents the peptide **CA18-Tn**. Peak at 22.5 min: starting material, peak at 23.7 min: Fmoc-PEG-TEV fragment **PEG_{27}- GDENLYFQ**. Based on LC peak area integration, the reaction proceeded to 64% conversion. (***B***) ESI-MS analysis of the **Fmoc-PEG_{27}-GDENLYFQ** peak at 23.7 min. Calculated mass for $C_{73}H_{118}N_{24}O_{27}S$: 2510.3 Da, found: 2511.7 Da $[(M+H]^+$. The second peak corresponds to a Na^+ adduct.

The crude TEV-digested material was used in the ligation reaction of figure S15 without further purification.

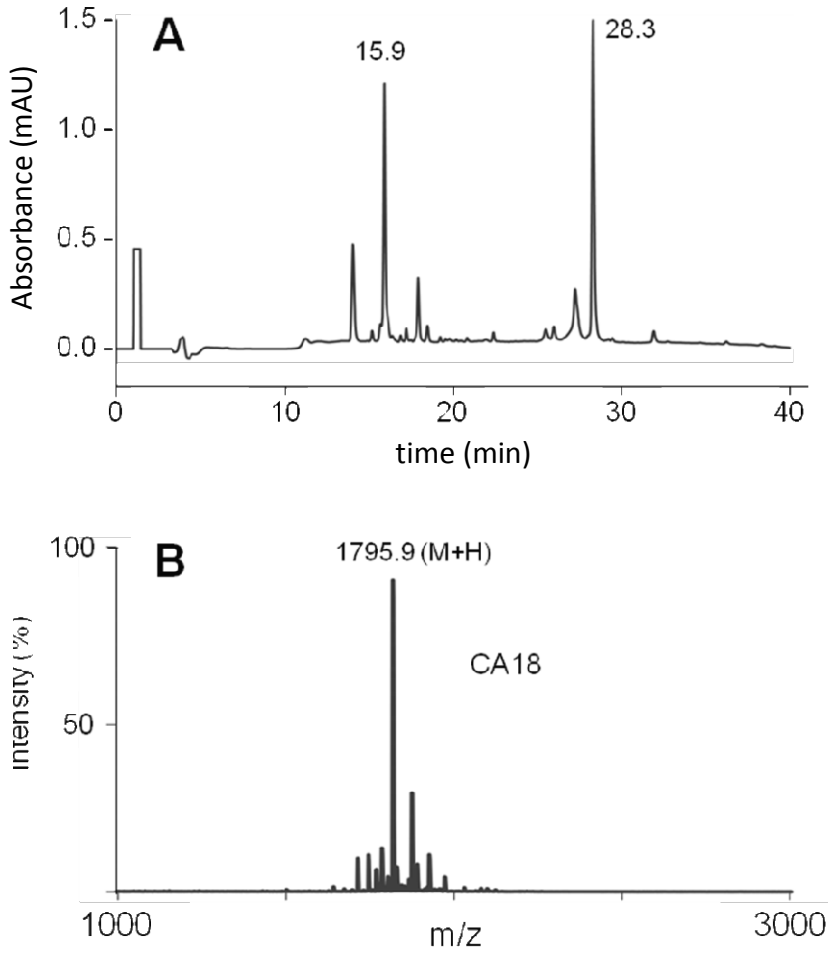


Figure S13. Deprotection and release of CA18 by reductive TEV digest of PTCA18*. (***A***) RP-HPLC (C18) profile of a 25 µg sample of a reaction containing 250 µg of the dithiomethane derivative **PTCA18*** digested with 12 µg TEV protease under reducing conditions (5 mM DTT). The peak at 27.2 min represents traces of undigested starting material, while the peak at 28.3 min contains the N-terminal fragment **PEG_{27}-GDENLYFQ**. The reaction proceeded to 90% conversion based on LC peak area integration. (***B***) MALDI-TOF MS spectrum of **CA18** eluting at 15.9 min (40% eluent B). Calculated mass for $C_{73}H_{118}N_{24}O_{27}S$: 1794.8 Da, found 1795.9 Da. Monoisotopic masses of positive ions $[M+H]^+$ are displayed.

Mass spectrometric analysis of O-glycosylation sites

O-glycosylation sites of the glycopeptides released from the PEGylated precursors were identified by alternating collision-induced dissociation (CID) and electron-transfer dissociation (ETD) ESI-MS/MS analyses on a Bruker HCTultra ETD II iontrap mass spectrometer as described above.

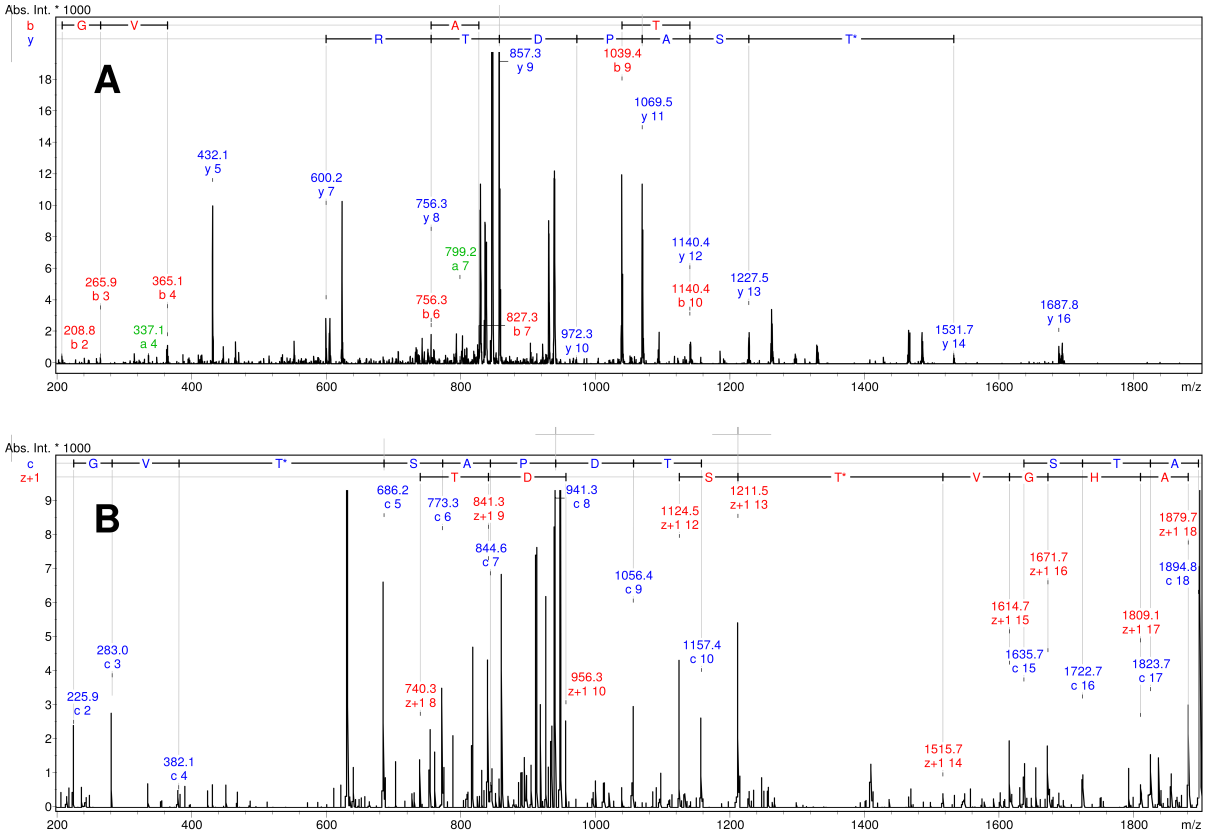


Figure S14. **O-glycans are attached to Thr 5 of A18. (*A*) Collision-induced dissociation and (*B*) Electron-transfer dissociation ESI-MS/MS analysis of A18-Tn.** Up to two doubly and triply charged precursor ions rising above a given threshold were used for alternating CID and ETD experiments. (***A)*** CID ESI-MS/MS analysis. Matched a- (*green*) b- (*red*) and y-ions (*blue*) are indicated above the peaks. The corresponding peptide sequence is displayed above the spectrum. The HexNAc modification (T*) was mapped based on the mass difference of the y13 (m/z 1227.504) and y14 ions (m/z 1531.718) derived from the *doubly charged precursor ion*. (***B***) ETD ESI-MS/MS analysis of A18-Tn. Matched z+1 and z+2 (*red*) and c-fragment (*blue*) ions are indicated in the spectra with the peptide sequence displayed above. The HexNAc modification of Thr 5 (T*) was mapped based on the mass difference between c4 (m/z 382.083) and c5 (m/z 686.24) of the c-series ions and the (z+1)13 (m/z 1211.490) and (z+1)14 ions (m/z 1515.682) derived from the *triply charged precursor ion*.

Native Chemical Ligation (NCL)

Native chemical ligations were performed in sodium phosphate or Tris-HCl buffered solutions with 50-250 mM 2-mercaptoethane sulfonate sodium salt as a ligation mediator. N-cysteinyl peptides were used at a concentration of 0.5 mM (100-250 µg scale) and 2.2 equivalents of the peptide-thioester were added. The reaction was monitored by MALDI-TOF MS and quantified by RP-HPLC after 1 h incubation at RT. After 20 hours, the reaction mixture was purified by RP-HPLC on a Vydac 218TP5415-C18 column (4.6 mm × 15 cm, 5 µm particle diameter, Grace Vydac) equipped with a 218GD54 guard (Grace Vydac) in a Beckman System Gold HPLC using 0.1% TFA and a gradient of 5-80% acetonitrile. Conversion was analysed based on HPLC peak integration.

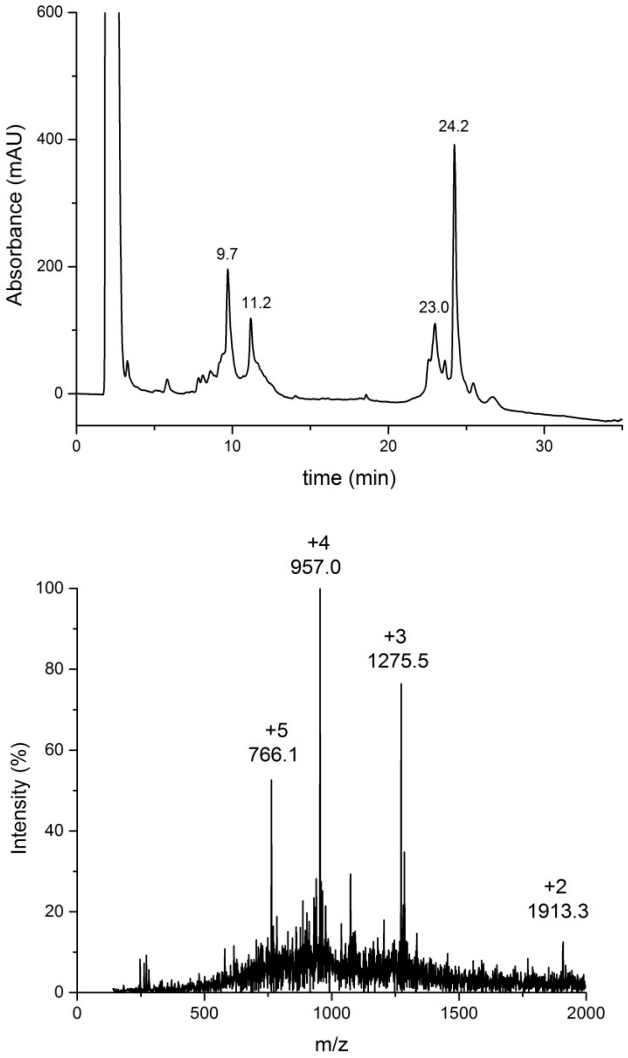


Figure S15. Native chemical ligation. (***A***) HPLC chromatogram of the one pot release-ligation reaction of peptide **CA18-Tn** (released from **PTCA18-Tn**, see figure S11**)** with the peptide-thioester **A20-SR**. (***B***) ESI-MS of the ligation product **A39-Tn** (peak at 11.2 min in ***A***). Calculated mass for $C_{160}H_{256}N_{50}O_{57}S$: 3823.9 Da, found: 1913.3 Da ($[M+2H]^{2+}$), 1275.5 Da ($[M+3H]^{3+}$), 957.0 Da ($[M+4H]^{4+}$) and 766.1 Da ($[M+5H]^{5+}$). The overall conversion to A39-Tn was 31%. The peak at 9.7 min represents the remaining peptide thioester **A20-SR**, the peak at 23.0 min is the educt **PTCA18-Tn**, the late eluting peak at 24.2 min contains the N-terminal Fmoc-PEG-TEV fragment **PEG_{27}-GDENLYFQ.**

ELISA using MAbs to *O*-glycan epitopes

ELISA experiments were performed in 96-well MaxiSorp plates (Nunc). The plates were coated with our panel of PEGylated peptide conjugates by incubation of 2 µg/mL dilutions in bicarbonate–carbonate buffer (pH 9.6) overnight at 4°C. Unspecific binding was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) that was followed by incubation with the respective MAbs for 2 h at room temperature. The MAbs used to analyze the O-glycoepitopes were 5F4 (anti-Tn), 3C9 (anti-T) and 114-2G11 (anti-GlcA) in comparison to the control antibody C595 with anti-MUC1 reactivity.[9-11] Antibody complexes were detected with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) as the secondary antibody. After TBS washing, the plates were developed with 3,3',5,5'-Tetramethylbenzidine (AppliChem) in citrate/acetate buffer, then the reaction was stopped with 100 mM H_2SO_4 and the signals were detected at a wavelength of 450 nm.
The monoclonal antibody 114-2G11 was originally raised against the *S. mansonii* circulating anodic antigen (C. Hokke, personal communication). In a separate study to be described elsewhere, this antibody was shown to specifically detect terminal glucuronic acid on isolated protein O-glycans. To our knowledge, no other MAb with GlcA-specificity has been described so far. Therefore, MAb 114-2G11 became an inevitable tool for the immunological studies of the glucuronyl-T antigen presented here.

References

1. E. Atherton and R. C. Sheppard, *Solid Phase Peptide Synthesis: A practical approach*, Oxford University Press, USA, 1989.
2. V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, *Analytical Biochemistry*, 1981, 117, 147-157.
3. T. Haack and M. Mutter, *Tetrahedron Letters*, 1992, 33, 1589-1592.
4. M. Mutter, A. Nefzi, T. Sato, X. Sun, F. Wahl and T. Wöhr, *Peptide Research*, 1995, 8, 145-153.
5. T. C. Manning, B. A. Schodin and D. M. Kranz, *Journal of Immunological Methods*, 1996, 192, 125-132.
6. E. P. Bennett, H. Hassan, U. Mandel, E. Mirgorodskaya, J. Burchell, J. Taylor-Papadimitriou, M. A. Hollingsworth, G. Merkx, A. G. van Kessel, H. Eiberg, R. Steffensen and H. Clausen, *Journal of Biological Chemistry*, 1998, 273, 30472-30481.
7. T. Schwientek, E. P. Bennett, C. Flores, J. Thacker, M. Hollmann, C. A. Reis, J. Behrens, U. Mandel, B. Keck, M. A. Schafer, K. Haselmann, R. Zubarev, P. Roepstorff, J. M. Burchell, J. Taylor-Papadimitriou, M. A. Hollingsworth and H. Clausen, *The Journal of Biological Chemistry*, 2002, 277, 22623-22638.
8. A. L. Sorensen, C. A. Reis, M. A. Tarp, U. Mandel, K. Ramachandran, V. Sankaranarayanan, T. Schwientek, R. Graham, J. Taylor-Papadimitriou, M. A. Hollingsworth, J. Burchell and H. Clausen, *Glycobiology*, 2006, 16, 96-107.
9. M. Thurnher, H. Clausen, N. Sharon and E. G. Berger, *Immunology Letters*, 1993, 36, 239-243.
10. H. Clausen, M. Stroud, J. Parker, G. Springer and S. Hakomori, *Molecular Immunology*, 1988, 25, 199-204.
11. M. R. Price, J. A. Pugh, F. Hudecz, W. Griffiths, E. Jacobs, I. M. Symonds, A. J. Clarke, W. C. Chan and R. W. Baldwin, *Br. J. Cancer*, 1990, 61, 681-686.