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Antibodies Directed Against GalNAc- and GlcNAc-*O*-Tyrosine Posttranslational Modifications – a New Tool for Glycoproteomic Detection

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Abstract: In the last decade, it was discovered that protein mucin-type *O*-glycosylation and *O*-GlcNAcylation modify Tyr residues besides the well explored Thr and Ser amino acids. Several glycoproteomic studies have identified α-GalNAc-*O*-Tyr modifications, and studies propose that β-GlcNAc-*O*-Tyr also exists as a new group of posttranslational modifications (PTMs). Specific bacterial toxins have further been identified to modify host GTPases with α-GlcNAc-*O*-Tyr to promote bacterial virulence. Despite being identified on numerous proteins, the biological roles, biosynthesis and expression of GalNAc- and GlcNAc-*O*-Tyr modifications are poorly understood. A major obstacle is the lack of tools to specifically detect and identify proteins containing these modifications. With this in mind, we prepared vaccine constructs and raised antibodies to enable selective detection of proteins carrying these new PTMs. The obtained polyclonal antibody sera were evaluated using ELISA and glycopeptide microarrays and were found to be highly selective for GlcNAc- and GalNAc-*O*-Tyr glycopeptides over the corresponding Ser- and Thr-modifications. For microarray analysis, synthetic GlcNAc- and GalNAc-*O*-Tyr Fmoc-amino acids were prepared and applied in Fmoc-SPPS to obtain an extensive *O*-glycopeptide library. After affinity purification, the antibodies were applied in western blot analysis and showed specific detection of α-GlcNAc-*O*-Tyr modified RhoA GTPase.

Introduction

Protein glycosylation is the most abundant and diverse form of all known post-translational modifications and plays key roles in many biological processes. As such, the specific glycosylation site is of great importance. Glycoproteomic analysis of *O*-linked glycosylation is highly challenging due to the complex and diverse glycan structures. Often, nearby and dense glycosylation sites make proteolytic cleavage and site specific analysis demanding, and in contrast to *N*-glycoproteomic workflows, endoglycosidases capable of efficient *O*-glycan release are not available. Because of their significant biological functions, "mucin-type" *O*-glycosylation (*O*-GalNAc-type) and *O*-GlcNAcylation (*O*-GlcNAc-type glycosylation) receive more and more attention from the glycoproteomic field. Whereas *O*-GlcNAcylation predominantly occurs on cytosolic, mitochondrial and nuclear proteins, and is usually not further elongated or modified to generate more complex glycan structures,[1] mucin-type *O*-glycosylation represents the most abundant and complex form of protein *O*-glycosylation and usually contains extension of the inner core GalNAc residue.[2] Since 2011, several studies reported that *N*-acetylhexosamine (HexNAc = α-GalNAc, α- or β-GlcNAc) does not only modify threonine (Thr) and serine (Ser) amino acid residues but also the hydroxyl group of the tyrosine (Tyr) side chain.[3] This new type of protein *O*-glycosylation was first discovered on Tyr-681 of the amyloid-β (Aβ) peptide the DAEFRHDSGYEVHHQK in the frame of a glycoproteomic study on CSF from Alzheimer's patients,[3a] and was in recent work found to influence the Aβ peptide aggregation properties.[3g] Due to glycan extension forming mono-, di- or tri-sialyl-core 1 ((α-Neu5Ac)$_{1\text{-}3}$-β-1,3-Gal-α-GalNAc−) structures, the identified peptides were considered to be modified with mucin-type *O*-glycosylation on the Tyr glycosylation site. Glycan analysis using "SimpleCell Strategy" and lectin weak affinity enrichment (LWAC) with the GalNAc specific lectin *Vicia villosa* agglutinin (VVA) enabled identification of additional GalNAc-*O*-Tyr modified peptides: Tyr-389 of the Nucleobindin-2 (NUCB2) peptide LEYHQVIQQMEQK, Tyr-50 of the Nucleobindin-1 (NUCB1) peptide GAPNKEETPATESPDTGLYYHR, Tyr-43 of the

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Extracellular matrix protein 1 (ECM1) peptide QLRPEHFQEVGYAAPPSPPLSR, Tyr-560 of the CD44 peptide RDPNHSEGSTTLLEGYTSHYPHTK and Tyr-113 of the proline-rich acidic protein-1 (PRAP1) peptide VLSPEPDHDSLYHPPPEEDQGEERPR.[3b,d] Glycoproteomic studies of isolated mitochondrial proteins led to the discovery of HexNAc-*O*-Tyr on Tyr-418 of the ATP synthase subunit beta (ATP5B) peptide IMDPNIVGNEHYDVAR, Tyr-186 of the voltage-dependent anion-selective channel protein 1 (VDAC1) peptide VTQSNFAVGYK and Tyr-96 of the aspartate aminotransferase (ASAT) peptide NLDKEYLPIGGLAEFCK.[3c] Due to glycopeptide affinity enrichment with the less selective lectin wheat germ agglutinin (WGA), it was not possible to conclude if the identified Tyr modified peptides contained a new PTM, β-GlcNAc-*O*-Tyr, or mucin-type *O*-glycosylation. However, the tissue localization and previously identified β-GlcNAc residues on nearby Ser and Thr sites would suggest a β-GlcNAc-*O*-Tyr modification.[4] Additionally, it has been reported that the *Photorhabdus asymbiotica* protein toxin (PaTox) modifies the switch II region of host GTPases, including the GTPases RhoA, Rac and Cdc42, with the PTM α-GlcNAc-*O*-Tyr, and thus modulates the GTPase activity.[3e,f,5] In this case, NMR was used to confirm the α-anomeric configuration after protein modification using UDP-GlcNAc as donor. Another study also reported about bacterial toxins that modify GTPases with Glc or GlcNAc on Ser- and Thr-sites.[5]

Because Ser and Thr are the expected acceptor amino acids, Tyr residues were previously not considered as potential glycosylation sites, and the often dense *O*-glycosylation on glycoproteins makes site-specific characterization challenging. The fact that Tyr modifications exist besides Ser- and Thr-HexNAcylation raises questions about their biosynthesis, expression and unique biological functions, which are unexplored to a large extend. The lack of efficient methods to determine the exact structures and linkages of the attached carbohydrate moieties, and to specifically detect and enrich these modifications makes the study of *O*-GalNAc- and *O*-GlcNAc-Tyr extremely challenging. Proper assignment of the HexNAc isomers in previous studies was in many cases not possible, and the glycoforms were assigned based on general knowledge about carbohydrate biosynthesis or lectin binding preferences. In line with this problem, we previously elucidated the use of a MS-based methodology for HexNAc isomer differentiation which allows site-specific characterization of *O*-GlcNAc and *O*-GalNAc on glycopeptides.[6] A recent study showed that several plant lectins used in LWAC recognize GalNAc on Tyr.[7] However, these lectins also detect *O*-GalNAc on Ser and Thr sites as well. Consequently, new tools for specific detection, enrichment and identification of *O*-GalNAc- and *O*-GlcNAc-Tyr need to be developed to gain insights into the functions of this new group of PTM.

Herein we present the generation of GlcNAc- and GalNAc-*O*-Tyr specific antibodies to selectively detect, profile and enrich peptides and proteins containing these modifications. Therefore, antigen glycopeptide-CRM conjugates with high α-GalNAc- or β-GlcNAc-*O*-Tyr density were generated to raise specific polyclonal antibodies, which were subsequently immunologically analyzed and evaluated by enzyme-linked immunosorbent assay (ELISA) and microarray binding experiments

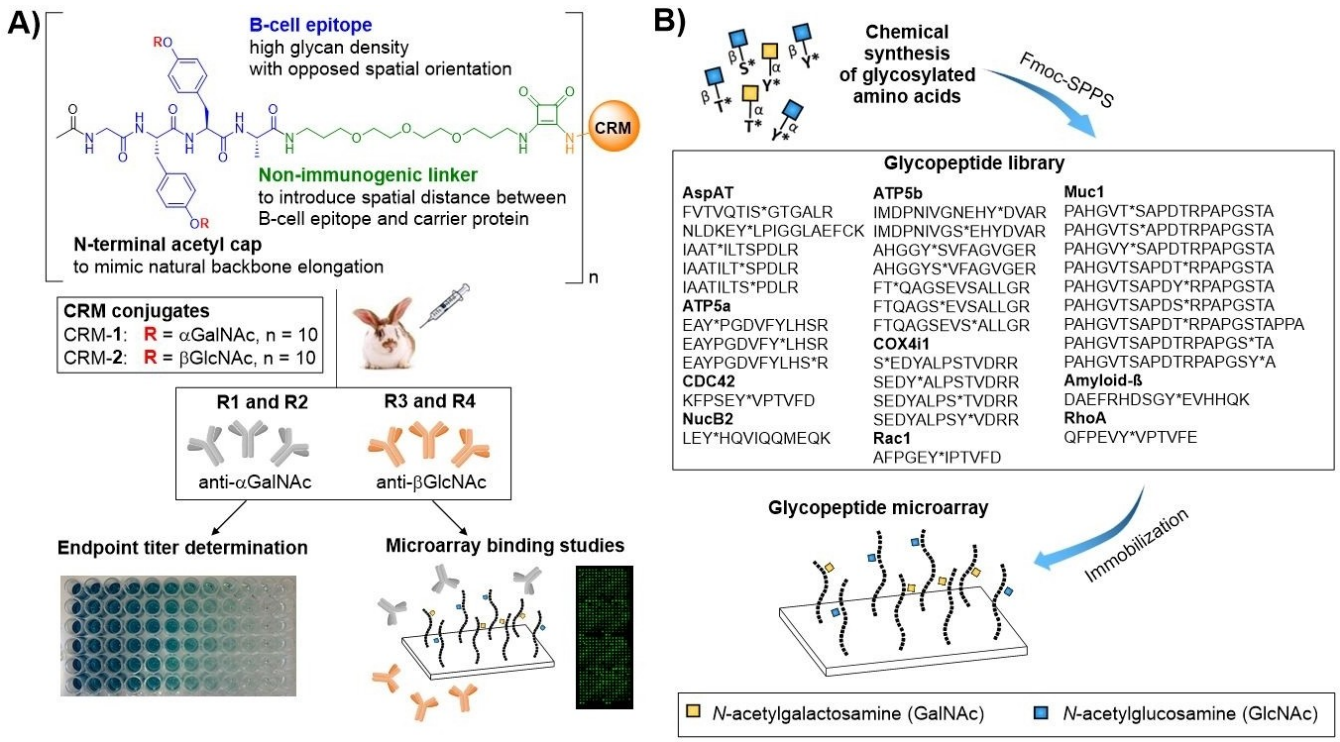


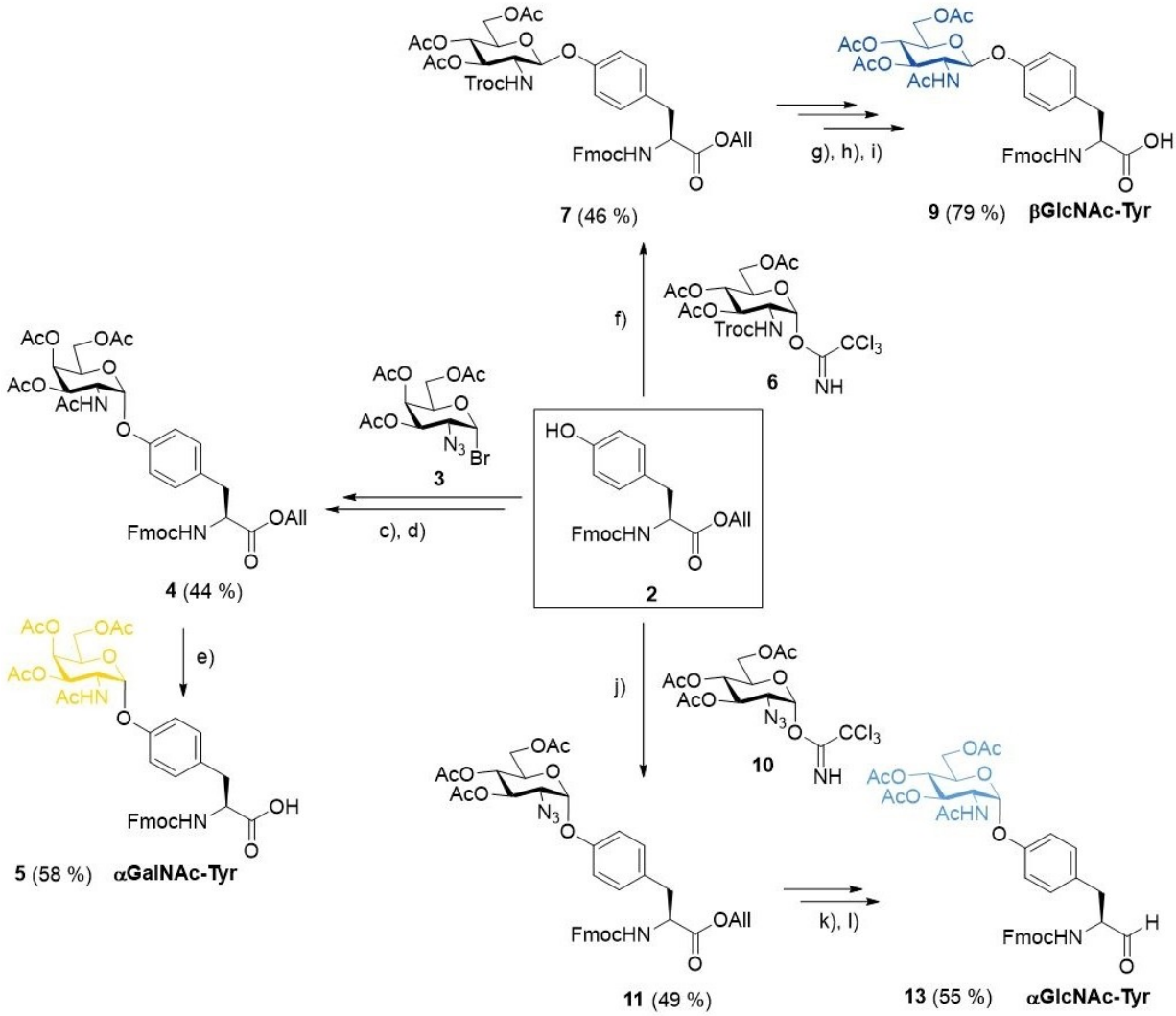
Figure 1. A) Preparation of antigen vaccines **CRM-1** and **CRM-2**, generation of polyclonal rabbit antibodies and evaluation of the obtained antibodies using ELISA and microarray binding studies; B) Synthesis of glycosylated amino acids, a (glyco-)peptide library and preparation of peptide microarrays.

(Figure 1A). To determine the antibody binding specificities, a peptide library was prepared that included peptide sequences carrying α-GalNAc, α- or β-GlcNAc on Ser, Thr or Tyr, and immobilized on microarrays (Figure 1B). Furthermore, the work aimed to explore if the obtained GalNAc- and GlcNAc-*O*-Tyr specific antibodies could be used to selectively detect this modification on protein level. Therefore, the antibodies were purified by affinity chromatography against GalNAc- and GlcNAc-*O*-Tyr antigen peptides, re-evaluated by ELISA and microarray assays and applied to specifically detect α-GlcNAc-*O*-Tyr modified RhoA, which was enzymatically modified using the *Photorhabdus asymbiotica* protein toxin (PaTox[G]), on a Western blot.

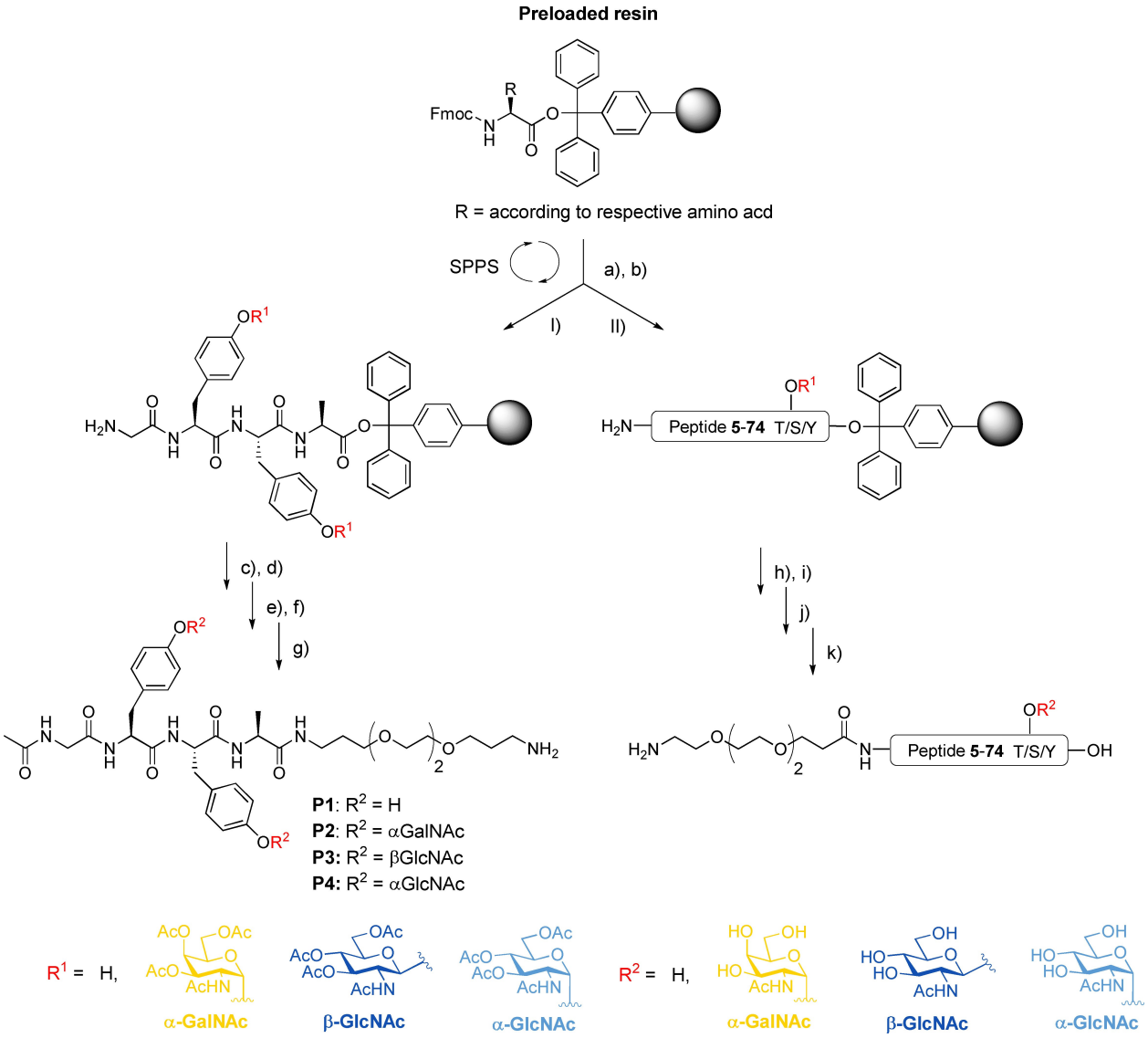
Results and Discussion

To prepare extensive GalNAc/GlcNAc-Tyr/Thr/Ser library containing 74 glycopeptides, glycosylated amino acid building blocks were assembled using common acceptor amino acids and corresponding glycosyl donors (Scheme 1), and subsequently incorporated into peptide sequences using Fmoc solid-phase peptide synthesis (Fmoc-SPPS) (Scheme 2).

The peracetylated α-GalNAc-Fmoc-Tyr building block **5** was synthesized using a similar strategy to previously described protocols preparing α-GalNAc-Fmoc-Ser/Thr amino acids (Scheme 1, Supplementary Scheme S1B).[8] Here, the Koenigs-Knorr method was used with $AgCO_3$ and $AgClO_4$ activation to couple the Fmoc-Tyr allyl ester **2** with the glycosyl bromide donor **3** bearing a non-participating azide to yield exclusively the α-isomer. The glycan azide function was converted to a



Scheme 1. Synthesis of the α-GalNAc-Tyr Fmoc amino acid building block 5, β-GlcNAc-Tyr Fmoc amino acid building block **9** and α-GlcNAc-Tyr Fmoc amino acid building block **13**. a) Fmoc-OSu, $NaHCO_3$, dioxane/H_2O (3 : 1), 0 °C–RT, 4 d; b) allyl bromide, DIPEA, THF, RT, overnight; c) $AgClO_4$, $AgCO_3$, MS 4 Å,DCM, 0 °C–RT, 2 d; d) AcSH, pyridine, RT, 72 h; e) $Pd(PPh_3)_4$, phenylsilane, THF, RT, 30 min; f) TMSOTf, MS 4 Å, DCM, 0 °C–RT, overnight; g) Zn, AcOH/THF (1 : 1), 50 °C, overnight; h) Ac_2O/pyridine (1 : 2), DMAP, RT, overnight; i) $Pd(PPh_3)_4$, phenylsilane, THF/DMF (1 : 1), RT, 1 h; j) TMSOTf, MS 4 Å, DCM/Et_2O (1 : 1), −20 °C, 2.5 h; k) AcSH, pyridine, RT, overnight; l) $Pd(PPh_3)_4$, phenylsilane, THF, RT, 30 min.



Scheme 2. Synthesis of glycopeptides I) **P1–P4** and II) **P5–P74**. a) Fmoc removal: 20% piperidine in DMF; b) Amino acid coupling: Fmoc-Xaa-OH (8.0 equiv.), HOBt (7.6 equiv.), HBTU (7.6 equiv.), DIPEA (16 equiv.) in DMF, 40 min; Fmoc-Ser(β-GlcNAc)-OH, Fmoc-Thr(β-GlcNAc)-OH, Fmoc-Thr(α-GalNAc)-OH, Fmoc-Tyr(β-GlcNAc)-OH, Fmoc-Tyr(α-GlcNAc)-OH or Fmoc-Tyr(α-GalNAc)-OH (1.5 equiv.), HATU (1.4 equiv.), HOAt (1.4 equiv.), DIPEA (3.0 equiv.) in DMF, 8 h; c) Capping: acetic anhydride, HOBt, DIPEA, DMF, 2 h; d) Release from resin: TFA/TIPS/H_2O (95 : 5 : 5), 2 h; e) Spacer coupling: *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine, HATU, HOAt, DIPEA, DMF, 4 h; f) Boc removal: dichloromethane/TFA (3 : 1), 4 h; g) Deprotection of the carbohydrates: 0.2 M NaOH/MeOH (pH 10.0), 18 h; h) Fmoc-triethylene glycol (3.0 equiv.), HBTU (2.9 equiv.), HOBt (2.9 equiv.), DIPEA (6.0 equiv.) in DMF, 2 h; i) Fmoc removal: 20% piperidine in DMF; j) Release from resin: TFA/TIPS/H_2O (95 : 5 : 5); Deprotection of the carbohydrates: MeONa/MeOH (pH 8.5–9.5) or 0.2 M NaOH/MeOH (pH 10.0).

NHAc group by reductive amidation to give **4** in 44% yield from compound **2**.[9] In the final step, the allyl ester group was removed using phenylsilane and $(Pd(PPh_3)_4$ catalysis to give α-GalNAc-Fmoc-Tyr-OH **5** in 58% yield.[10] To generate the β- and α-GlcNAc-Fmoc-Tyr building blocks **9** and **13**, the tyrosine acceptor amino acid **2** was glycosylated with trichloroacetimidate glycosyl donors **6** and **10**, respectively (Scheme 2, Supplementary Scheme S1C and D).[11] The formation of the glycosidic linkage was controlled by either introducing a participating *N*-Troc at C-2 to promote β-glycosylation, or by installing a non-participating azide function to promote α-glycosylation, yielding **7** and **11** in 46% and 49%, respectively.

The *N*-Troc group of **7** was then converted to an acetamide under reductive conditions using Zn/AcOH, followed by acetylation of the free amine to obtain **8** in 97% yield. The glycan azido group of **11** was reduced to the corresponding acetamide using thioacetic acid in pyridine to give **12** in 70% yield. The *C*-terminal allyl esters of **8** and **12** were removed to obtain the β- and α-GlcNAc-Fmoc-Tyr building blocks **9** and **13**, in 81% and 79% yield, respectively. The obtained peracetylated GalNAc/GlcNAc-Fmoc-Ser/Thr/Tyr building blocks were then used in Fmoc-SPPS to prepare α-GalNAc-*O*-Tyr and β-GlcNAc-*O*-Tyr antigen peptide-CRM vaccine conjugates as well as a library of glycopeptides carrying α-GalNAc, α- or β-GlcNAc on Ser, Thr or Tyr for microarray binding studies. Therefore, antigen

glycopeptides **P1**–**P4** were generated containing short peptide sequences with high α-GalNAc- or β-GlcNAc-O-Tyr density, respectively, to serve as B-cell epitopes (Figure 1A and Scheme 2). The antigen peptides were conjugated to CRM^{197}, which was used as carrier protein to elicit strong immune responses,[12] by using a non-immunogenic triethylene glycol linker[13] to obtain the corresponding vaccine constructs **CRM-1** and **-2**. The glycopeptide library was synthesized by automated Fmoc-SPPS. Standard Fmoc amino acids were coupled using HBTU/HOBt (Scheme 2). Glycosylated amino acids were pre-activated using the more reactive HATU/HOAt and manually added to the resin and the coupling time was extended to 8 h.[14] To prepare antigen glycopeptides **P1**–**P4**, the *N*-terminus was acetylated after complete peptide assembly. The peptides were released from the resin and a non-immunogenic linker was introduced to the free *C*-terminus for either subsequent conjugation to an immune carrier protein, or for immobilization on microarray slides.[15] The *tert*-butyloxycarbonyl (Boc) protecting group on the linker and the *O*-acetyl protecting groups on the glycans were removed and glycopeptides **P1**–**P4** were obtained after purification by preparative HPLC. To construct vaccine conjugates for immunization experiments and ELISA antibody endpoint titer determination, antigen peptides **P2** and **P3** were conjugated to CRM^{197} or BSA as carrier proteins, respectively. CRM^{197}, which is a non-toxic mutant of diphtheria toxin, was chosen as immune carrier protein since it has successfully been used for immune stimulation with various glycoconjugates.[12] Glycopeptides **P2** (α-GalNAc-*O*-Tyr) and **P3** (β-GlcNAc-*O*-Tyr) were coupled to diethyl squarate, generating the corresponding squaric acid monoamides **14** and **15** (Supplementary Figure S1). Finally, compounds **14** and **15** were conjugated to CRM^{197} or bovine serum albumin (BSA) to obtain the α-GalNAc-*O*-Tyr conjugates **CRM-1** and **BSA-1**, and β-GlcNAc-*O*-Tyr conjugates **CRM-2** and **BSA-2**. The glycopeptide-protein conjugates were purified by ultrafiltration and the loading rates were determined by MALDI-TOF mass spectrometry (Supplementary Figure S2–S5). To induce antibodies against antigens α-GalNAc-Tyr and β-GlcNAc-Tyr, two rabbits were immunized with **CRM-1** (rabbit **R1** and **R2**) or **CRM-2** (rabbit **R3** and **R4**), respectively. The obtained antibody sera were analyzed by ELISA and microarray binding experiments.

For ELISA experiments, 96-well microtiter plates were coated with BSA conjugates **BSA-1** or **BSA-2**, as well as with BSA only to determine the cutoff value. The rabbit sera were added to the wells at 2-fold dilutions and subsequently probed with a secondary antibody, and streptavidin-conjugated horseradish peroxidase (HRP) was used for colorimetric detection. The endpoint titers of the ELISAs were determined to be approximately 2 024 000 and 4 096 000 for rabbits **R1** and **R2**; and 4 096 000 and 2 048 000 for rabbits **R3** and **R4**, respectively (Supplementary Figure S13). These findings indicate high concentrations of specific antibodies in the rabbit sera.

To evaluate the binding specificities of the obtained rabbit antisera, a peptide library consisting of 74 structurally well-defined synthetic glycopeptide sequences carrying α-GalNAc, α- or β-GlcNAc on Tyr, Ser or Thr was prepared (Scheme 2, Supplementary Table S1). This library contained different glycopeptide *O*-GalNAc and GlcNAc isomers, and included synthetic sequences of amyloid-β, Nuclobindin-2, previously identified by VVA LWAC enrichment and HCD-LC-MS as GalNAc-*O*-Tyr modified tryptic glycopeptide fragments, and isomeric glycopeptides of the human host RhoA, Rac1 and CDC42 GTPases, which were previously identified to be modified with α-GlcNAc-Tyr by bacterial toxins.[3a,b,e] Synthetic glycopeptide *O*-GalNAc and GlcNAc isomers were generated of previously identified tryptic glycopeptides of ATP5B and ASAT.[3c] Glycopeptides with identified nearby β-GlcNAc-Ser or -Thr sites of ATP5B and ASAT were synthesized including glycan isomer analogs modified at the specific site, or nearby potential glycosylation sites. Mitochondrial glycopeptide sequences of ATP synthase alpha (ATP5A) and Cytochrome c oxidase subunit 4 isoform 1 (Cox4i1) were synthesized, which were previously identified to contain β-GlcNAc-Ser or -Thr sites, and contained nearby Tyr sites.[4] Additionally, analogs of the MUC1 tandem repeat sequence were generated by replacing known and well explored GalNAc-Ser/Thr sites with HexNAc-Tyr to further explore the influence of Tyr modifications at different glycosylation sites on antibody recognition.[16] Peptides **P5**–**P74** were synthesized by Fmoc-SPPS according to the strategy described above. After complete peptide assembly, a triethylene glycol spacer was introduced to the *N*-terminus to later covalently bind the peptides to the microarray (Scheme 2). After assembly, the peptides were cleaved from the resin with simultaneous removal of acid-sensitive protecting groups on the amino acid side chains. The *O*-acetyl protecting groups on the glycans were removed by sodium methoxide treatment, and peptides **P1**–**P74** were obtained after purification by preparative HPLC.

The synthetic peptides **P1**–**P74** were immobilized on NHS-activated hydrogel slides (Nexterion® slide H, Schott) by microarray spotting and unreacted NHS groups were blocked with ethanolamine. The immobilized glycopeptides were then used to evaluate the specificity of the raised polyclonal antibodies for α-GalNAc, α- or β-GlcNAc-Tyr glycopeptides in comparison with the corresponding Ser and Thr analogs. To adjust for variations in antibody titers, the microarrays were incubated with a dilution series of the anti-α-GalNAc-*O*-Tyr rabbit antisera **R1**, **R2** and the anti-β-GlcNAc-*O*-Tyr rabbit antisera **R3**, **R4**. The rabbit antibodies were detected by a secondary biotinylated goat anti-rabbit IgG, followed by treatment with Cy5-labeled streptavidin, and the fluorescence was read out. Microarray analysis showed differences in binding of the rabbit sera towards the glycopeptides regarding different glycosylation sites and different GalNAc or GlcNAc isomers at the same glycosylation site. The microarray binding data for rabbit sera **R1**, **R2**, **R3** and **R4** are shown in Supplementary Figure S19–S21 and surface dissociation constants (Surf. K_D) are given in Supplementary Table S2. The microarray data showed that all rabbit antisera exhibited a high affinity for GlcNAc- and GalNAc-*O*-Tyr glycopeptides. In contrast, the corresponding Ser and Thr peptides were not recognized. Only at high serum concentrations, weak binding to β-*O*-GlcNAc on Ser of rabbit antisera **R2** and **R3** was observed, but not for rabbit sera **R1** and **R4** (Figure 2A). While weak affinity of rabbit sera **R3, R1**, and **R4** was observed for the unglycosylated antigen structure **P1**, none of the other

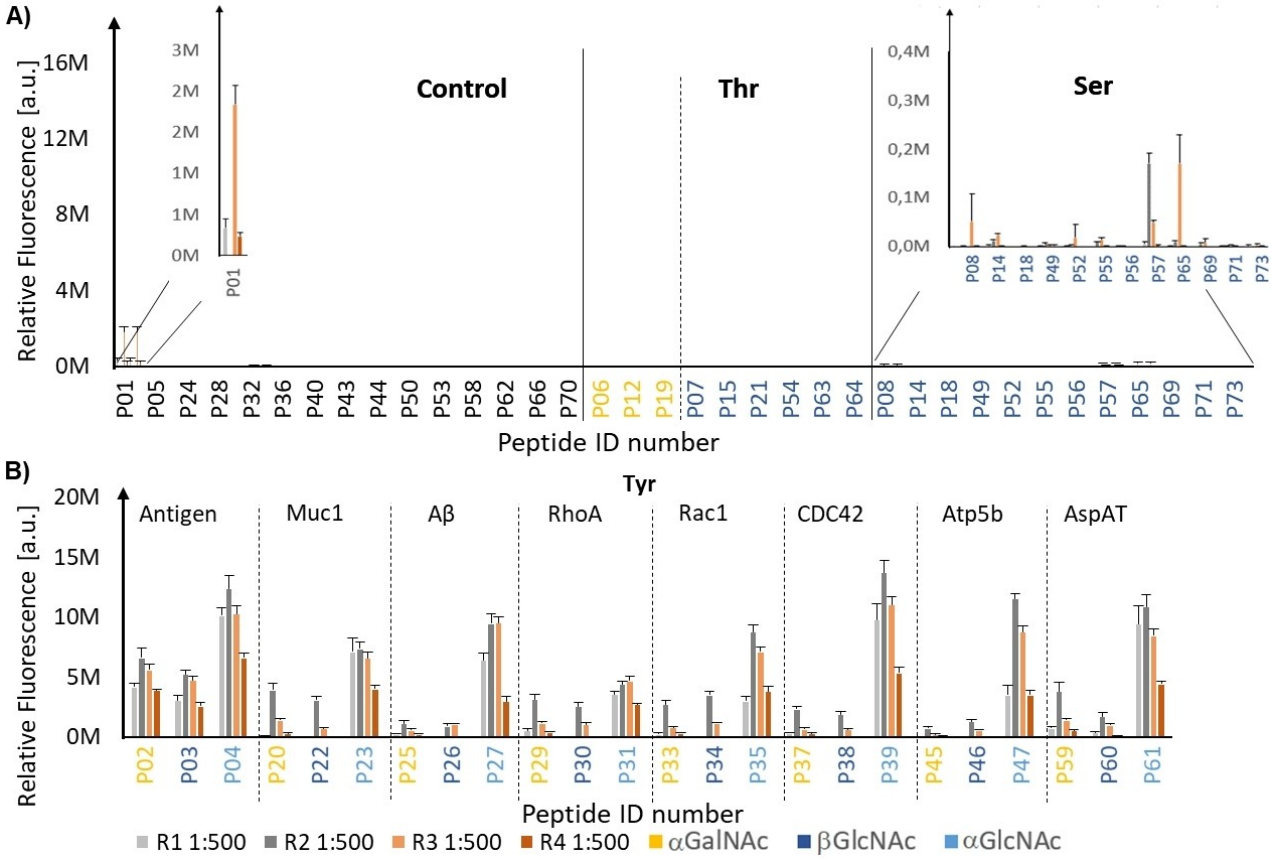


Figure 2. Binding of anti-α-GalNAc (**R1** and **R2**) and anti-β-GlcNAc (**R3** and **R4**) rabbit sera at dilution 1 : 500 to A) unglycosylated peptides and α-GalNAc-Thr or β-GlcNAc-Thr/Ser glycopeptides; B) α-GalNAc-, β-GlcNAc-, or α-GlcNAc-Tyr glycopeptides.

unglycosylated peptides were recognized. Surprisingly, all rabbit antisera exhibited cross-reactivity between the different HexNAc-*O*-Tyr isoforms indicating that the raised antibodies tolerate minor structural conformational differences between closely related monosaccharide structures (Figure 2B). Among the HexNAc-*O*-Tyr isomers, the antibodies showed binding preferences for α-GlcNAc-*O*-Tyr over the corresponding α-GalNAc and β-GlcNAc-*O*-Tyr peptides irrespective of whether or not the antibodies were directed against the α-GalNAc or β-GlcNAc antigen peptide. In order to obtain monospecific α-GalNAc-, α-GlcNAc- and β-GlcNAc-*O*-Tyr antibodies, rabbit serum **R2** was purified by affinity enrichment against the antigen glycopeptides α-GalNAc-*O*-Tyr **P2**, β-GlcNAc-*O*-Tyr **P3** and α-GlcNAc-*O*-Tyr **P4**. Rabbit serum **R2** was chosen for affinity purification because it exhibited the strongest binding towards α-GalNAc and β-GlcNAc on Tyr, and binding towards α-GlcNAc-*O*-Tyr peptides was comparable with rabbit serum **R3** (Supplementary Table S2). Additionally, the serum had a high endpoint titer and it was the only serum that did not recognize the unglycosylated antigen peptide **P1**. Polyclonal anti-α-GalNAc antibodies were purified from rabbit serum **R2** using affinity chromatography columns prepared by coupling of the immunogenic peptides **P2** (α-GalNAc-*O*-Tyr), **P3** (β-GlcNAc-*O*-Tyr) and **P4** (α-GlcNAc-*O*-Tyr) to HiTrap NHS-Activated HP affinity columns, and affinity purified (AP) antibodies **AP-1**, **AP-2** and **AP-3** were obtained, respectively. Next, the total protein concentrations of the purified antisera were quantified by 280 nm absorption measurements. Additionally, the enriched sera were analyzed for purity by SDS-gel electrophoresis. SDS-PAGE analysis of the purified rabbit **R2** antibodies showed a single band indicative of the desired purity (Supplementary Figure S23). Then, the endpoint titers were determined by ELISA. To also obtain the endpoint titer for the AP-α-GlcNAc-O-Tyr antibodies **AP-3**, the α-GlcNAc-Tyr-BSA conjugate **BSA-3** was generated. Therefore, glycopeptide **P4** was coupled to diethyl squarate, generating the corresponding squaric acid monoamide **16**. In the next step, **16** was conjugated to BSA to obtain the desired conjugate **BSA-3**. The glycopeptide-protein conjugate was purified by ultrafiltration and the peptide to protein loading ratio was determined by MALDI-TOF mass spectrometry (Supplementary Figure S6). ELISA analysis showed that all purified antibodies exhibited good immunoreactivity with the antigen conjugates α-GalNAc-*O*-Tyr **BSA-1**, β-GlcNAc-*O*-Tyr **BSA-2** and α-GlcNAc-*O*-Tyr **BSA-3**. End-point titers of purified antibodies for ELISA experiments were determined to be in a low ng/mL range (0.34–5.64 ng/mL) for AP-α-GalNAc-Tyr antibodies **AP-1**, AP-β-GlcNAc-Tyr antibodies **AP-2** and for AP-α-GlcNAc-Tyr antibodies **AP-3**, respectively (Supplementary Figure S24). These titers indicate that high concentrations of

specific high-affinity antibodies could be enriched during the affinity purifications.

Then, the binding specificities of the purified antibodies were evaluated using the glycopeptide microarray library. The microarray binding data for affinity purified antibodies **AP-1**, **AP-2**, and **AP-3** are shown in Figure 3 and Supplementary Figures S14–S16, and surface dissociation constants (Surf. K_D) are given in Supplementary Table S3. As desired, all purified antibodies showed no cross-reactivity towards the unglycosylated peptides, or Ser and Thr glycopeptides (Figure 3A). The affinity enriched antisera **AP-1**, **AP-2**, and **AP-3** displayed similar binding patterns to the different GalNAc- and GlcNAc-*O*-Tyr glycopeptides (Figure 3B) However, the affinity purifications did not result in a dramatic improvement of antibody selectivity towards the particular HexNAc-*O*-Tyr antigen immobilized on the affinity column. Instead, the higher affinity to the α-GlcNAc-*O*-Tyr over the α-GalNAc-*O*-Tyr and β-GlcNAc-*O*-Tyr glycopeptides remained (Figure 3, Supplementary Table S3).

Additionally, we evaluated a commercial *O*-β-GlcNAc specific monoclonal mouse antibody (CTD 110.6), which is commonly used to detect Ser- and Thr-*O*-GlcNAcylation. The determined mAb binding specificities towards *O*-β-GlcNAc-Ser, -Thr and -Tyr were compared with our raised Tyr specific rabbit antisera (Figure 3). While the *O*-GlcNAc mAb was highly selective for peptides modified with *O*-β-GlcNAc, surprisingly not all peptides *O*-β-GlcNAcylated on Ser and Thr peptides were recognized. However, the monoclonal antibody bound to all β-GlcNAc-*O*-Tyr peptides. Peptides glycosylated on Tyr were stronger recognized than the respective Ser and Thr glycopeptides indicating that hydrophobic interactions with the Tyr residue increases the binding drastically. Additionally, we could observe that our affinity purified antibodies **AP-1** to **-3** show binding intensities comparable with the monoclonal anti-*O*-β-GlcNAc antibody at similar antibody concentrations. These findings suggest that our anti-HexNAc-*O*-Tyr antibodies can be used for bioanalytical applications using similar antibody concentrations as for the commercial monoclonal mouse anti-

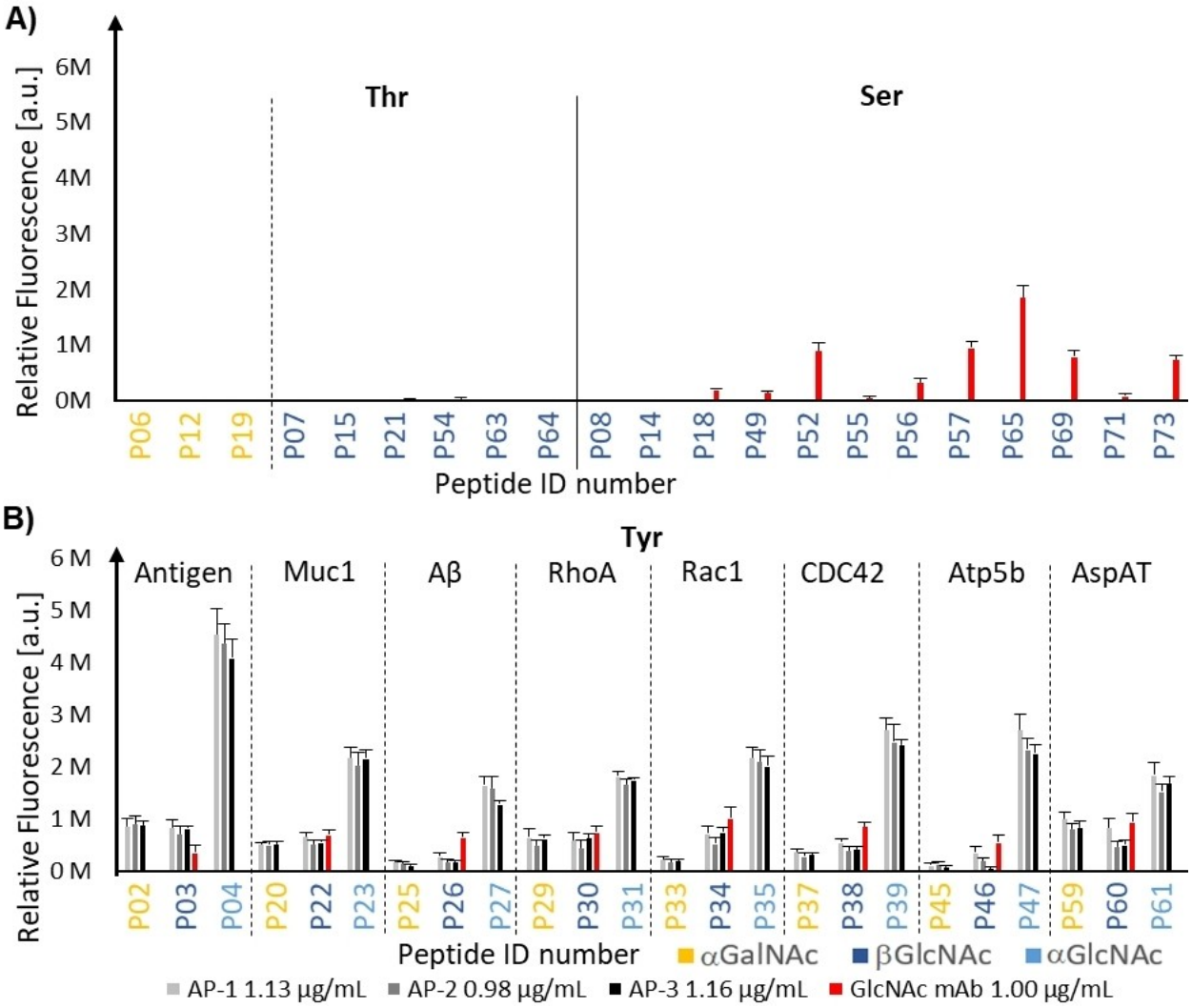


Figure 3. Binding of affinity purified rabbit antibodies **AP-1**, **AP-2**, **AP-3** and anti-*O*-βGlcNAc mAb CTD 110.6 at concentrations 1.13 µg/mL, 0.98 µg/mL, 1.16 µg/mL and 1.00 µg/mL, respectively to A) α-GalNAc-Thr or β-GlcNAc-Thr/Ser glycopeptides; B) α-GalNAc-, β-GlcNAc-, or α-GlcNAc-Tyr glycopeptides.

body. The affinity enriched HexNAc-*O*-Tyr antibody sera **AP-1, -2** or **-3**, which are selective towards Tyr modified glycopeptides, and the β-GlcNAc selective monoclonal anti-*O*-β-GlcNAc antibody CTD 110.6 exhibit affinities that complement each other. In a combined format, these antibodies would enable selective detection of β-GlcNAc-*O*-Tyr modified glycopeptides.

Furthermore, the ability of the affinity enriched HexNAc-*O*-Tyr antibody sera **AP-1, -2** or **-3** to recognize tyrosine HexNAcylation on different MUC1-BSA conjugates was tested in ELISA experiments. First, MUC1 glycopeptides **P6** to **P8**, which were glycosylated with β-GlcNAc or α-GalNAc on serine or threonine, were coupled to diethyl squarate to generate the corresponding squaric acid monoamides **17** to **19**. Then, the monoamides were conjugated to BSA, respectively, to obtain the conjugates **BSA-4** (α-GalNAc-*O*-Thr), **BSA-5** (β-GlcNAc-*O*-Thr), and **BSA-6** (β-GlcNAc-*O*-Ser). Then, MUC1 glycopeptides **P9** to **P11** glycosylated with the different HexNAc moieties on tyrosine were used to prepare the squaric acid monoamides **20** to **22**. Subsequently, the squaric acid conjugates were conjugated to BSA to generate **BSA-7** (α-GalNAc), **BSA-8** (β-GlcNAc) and **BSA-9** (α-GlcNAc). All glycopeptide-protein conjugates were purified by ultrafiltration and the peptide to protein loading ratio was determined by ESI-TOF mass spectrometry (Supplementary Figures S7 to S12). ELISA analysis showed that all purified antibodies showed good binding to HexNAc-*O*-Tyr-BSA conjugates **BSA-7** to **BSA-9** with (Surf. $K_D = 9$ ng/mL to 100 ng/mL) with the α-GlcNAc-*O*-Tyr conjugate **BSA-9** being the best binder for all purified antibodies (Supplementary Figure S25). While **AP-1** (AP-α-GalNAc-*O*-Tyr) and **AP-3** (AP-α-GlcNAc-*O*-Tyr) showed a preference for the α-GalNAc-*O*-Tyr conjugate **BSA-7** over the β-GlcNAc-*O*-Tyr conjugate **BSA-8**, **AP-2** (AP-β-GlcNAc-*O*-Tyr) bound to both BSA conjugates equally well. As expected, the BSA conjugates **BSA-4** to **BSA-6** carrying HexNAcylation on serine or threonine residues were only weakly recognized by the antibody sera **AP-1, or -2** at high concentrations. However, **AP-3** (purified against the α-GlcNAc antigen peptide) showed stronger binding to **BSA-4** to **BSA-6** (Surf. $K_D = 581$ ng/mL to 646 ng/mL).

Finally, we applied our purified antibodies to specifically detect HexNAc-*O*-Tyr modifications on protein level by western blot analysis. Therefore, SDS gel electrophoresis of BSA (negative control), BSA conjugates **BSA-4** to **-9** and α-crystalline, which carries β-GlcNAc-Ser/Thr modifications, was performed (Supplementary Figure S26). Afterwards, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and probed with purified rabbit antibodies **AP-1**, **AP-2** and **AP-3**. The resulting protein-antibody complexes were detected with a secondary labeled antibody (donkey anti-rabbit IgG Alexa Fluor 488 conjugate) and scanned with a fluorescence imaging system (Figure 4 and Supplementary Figures S27 and S28). Western blot analysis showed that none of the affinity purified antibodies detected the unmodified BSA (lane 2), or the β-GlcNAc-modified α-crystalline (lane 9). In agreement with the microarray and ELISA experiments, all antibodies detected BSA conjugates **BSA-7** to **BSA-9** (lanes 6 to 8), which were glycosylated on tyrosine, with the α-GlcNAc-*O*-Tyr conjugate **BSA-9** (lane 8) being the best binder. Here, **AP-1** (AP-α-GalNAc-*O*-Tyr) and **AP-2** (AP-β-GlcNAc-*O*-Tyr) specifically bound to BSA conjugates modified with HexNAc-*O*-Tyr, **AP-3** (AP-α-GlcNAc-*O*-Tyr) again weakly recognized BSA conjugates **BSA-4** to **BSA-6** that were HexNAcylated on serine or threonine. These results together with the finding from the ELISA experiment indicate that **AP-3** is less specific for HexNAc-*O*-Tyr than the other purified antibodies and consequently might in contrast to **AP-1** and **AP-2** not be applicable to specifically detect HexNAc-*O*-Tyr modifications on proteins.

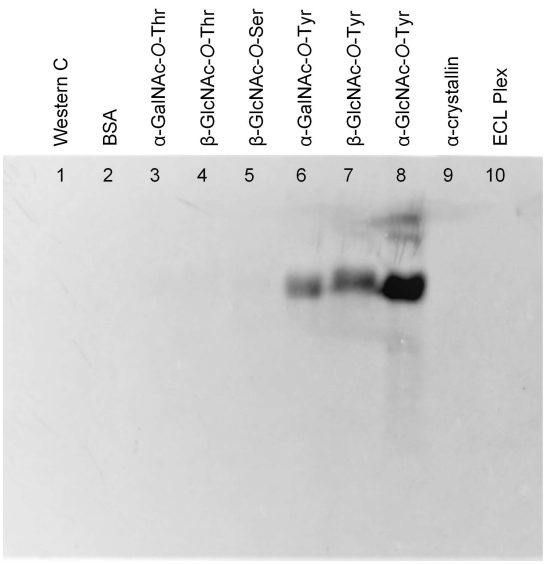


Figure 4. Western blot of BSA (lane 2), **BSA-4** (lane 3), **BSA-5** (lane 4), **BSA-6** (lane 5), **BSA-7** (lane 6), **BSA-8** (lane 7), **BSA-9** (lane 8) and α-crystallin (lane 9) probed with HexNAc tyrosine specific antibody **AP-1** (AP-α-GalNAc). Protein standards in lanes 1 and 10.

Since all affinity purified antibodies exhibited strong binding to α-GlcNAc-*O*-Tyr modified peptides and BSA conjugates, we applied them to detect α-GlcNAcylation on the small GTPase RhoA, which is known to be glycosylated on tyrosine by the *Photorhabdus asymbiotica* protein toxin.[3e,f] Therefore, we enzymatically modified RhoA with α-GlcNAc using UDP-GlcNAc and the glycosyltransferase domain of PaToxA (PaToxA^G). After enzyme treatment, SDS gel electrophoresis was performed with α-GlcNAcylated RhoA and unmodified RhoA (Supplementary Figure S29). Then, the proteins were transferred to PVDF membrane and probed with the purified rabbit antibodies **AP-1**, **AP-2** and **AP-3**. After incubation with the previously mentioned secondary labeled antibody, the membranes were scanned with a fluorescence imaging system (Figure 5 and Supplementary Figure S30). Whereas the unmodified RhoA was not recognized, the α-GlcNAcylated RhoA was successfully detected with our HexNAc-*O*-Tyr specific rabbit antibodies. These data suggest that we generated HexNAc-*O*-Tyr specific polyclonal rabbit antibodies **AP-1**, **AP-2** and **AP-3** that with high affinity detect α-GlcNAc-*O*-Tyr modified proteins.

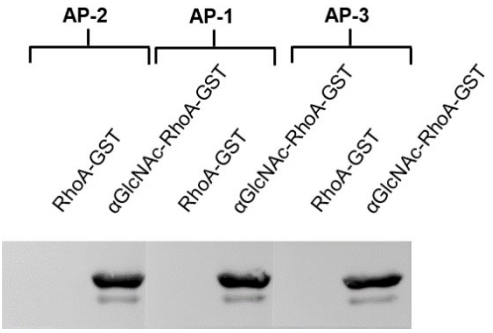


Figure 5. Binding Western blot of RhoA-GST (lanes 1, 3 and 5) α-GlcNAc-RhoA-GST (lanes 2, 4 and 6) probed with HexNAc Tyr specific antibodies **AP-1** (lanes 3 and 4), **AP-2** (lanes 1 and 2) and **AP-3** (lanes 5 and 6). The affinity purified antibodies bound to α-GlcNAcylated RhoA, whereas the unmodified RhoA-GST control was not detected on the Western blot. The lower band indicates partial protein breakdown of α-*O*-GlcNAcylated RhoA.

Conclusion

In summary, we generated polyclonal rabbit antibodies that are highly specific for α-GalNAc-, β-GlcNAc- and α-GlcNAc-*O*-Tyr glycopeptides, and can be applied to detect this relatively new group of protein modifications on protein level. Furthermore, a methodology was developed to efficiently synthesize GalNAc- and GlcNAc-*O*-Tyr amino acid building blocks, which were subsequently applied in Fmoc-SPPS to generate α-GalNAc- and β-GlcNAc-Tyr antigen glycopeptides. These antigen peptides were then conjugated to CRM[197] and the obtained vaccine constructs were used to immunize rabbits to produce HexNAc-*O*-Tyr specific antibody sera. The sera were evaluated using ELISA and microarray binding assays. Consequently, we generated GalNAc- and GlcNAc-*O*-Tyr antigen peptide BSA conjugates for ELISA assays as well as a library of 74 synthetic unglycosylated and α-GalNAc-, β-GlcNAc- and α-GlcNAc-*O*-Tyr/Ser/Thr glycopeptides, which were subsequently printed on microarray slides. Microarray binding studies with the polyclonal rabbit sera showed that the raised antibodies exhibited strong binding to all HexNAc isoforms with a higher affinity for α-GlcNAc-*O*-Tyr glycopeptides. In contrast, low or no binding affinities were observed for unglycosylated and glycosylated Ser or Thr peptides. To evaluate abilities of the obtained antibodies to selectively detect this new protein modification in more complex samples, we affinity purified a rabbit serum with a high titer and high affinities for all HexNAc *O*-Tyr glycopeptide isomers against antigen peptides, and re-evaluated the obtained purified antibodies via microarray analysis and ELISA experiments. To test their ability to detect HexNAc-*O*-Tyr on protein level, we generated MUC1-BSA conjugates carrying α-GalNAc, β-GlcNAc or α-GlcNAc on tyrosine, serine or threonine, and applied them in western blot analysis. All antigen-purified antibodies showed strong recognition of BSA conjugates modified with HexNAc-*O*-Tyr and no, or only weak recognition of BSA conjugates glycosylated on serine or threonine. Since the affinity purified antibodies exhibited a strong preference for α-GlcNAc-*O*-Tyr, we enzymatically modified the host GTPase RhoA with α-GlcNAc-*O*-Tyr using the bacterial toxin PaToxA[G] and blotted the obtained conjugate as well as the unmodified RhoA. Whereas the unmodified protein was not recognized by our purified rabbit antibodies, α-GlcNAc-*O*-Tyr-RhoA was successfully detected. In conclusion, we generated HexNAc-*O*-Tyr specific polyclonal rabbit antibodies that can detect α-GlcNAc-*O*-Tyr modified proteins. These findings support our hypothesis that the affinity purified antibodies can be applied to detect HexNAc-*O*-Tyr glycosylation in biological samples. Additionally, the *O*-β-GlcNAc specific monoclonal mouse antibody (CTD 110.6), which is commonly used for detection of Ser- and Thr *O*-GlcNAcylation by the glycobiology community, was evaluated on glycopeptide microarrays and found to also recognize β-GlcNAc-*O*-Tyr modifications. In conclusion, the affinity enriched HexNAc-*O*-Tyr antibodies **AP-1**, **AP-2** and **AP-3** and the *O*-β-GlcNAc mAb CTD 110.6 are new tools available to explore the glycobiology behind α-GalNAc-, β-GlcNAc- and α-GlcNAc-*O*-Tyr protein glycosylations.

Experimental Section

Supporting information for this article is given via a link at the end of the document.

General methods: All general methods in this work are described in Section 1.1 in the Supporting Information.

Synthesis of HexNAc-*O*-Tyr amino acids: Synthesis procedures and NMR-data for the HexNAc-*O*-Tyr amino acids are described in sections 1.1 to 1.7 in the Supporting Information.

Synthesis of the α-GalNAc-Tyr amino acid building block

Compound 4: A solution of Fmoc-tyrosine-O-allyl ester **2** (1.56 g, 3.50 mmol, 1.0 equiv.) in dry dichloromethane (30.0 mL) was added to a dry flask containing molecular sieve (1.81 g, 4 Å) under inert atmosphere. The suspension was cooled in an ice-bath and stirred for 1 h. Meanwhile, silver perchlorate (236 mg, 1.05 mmol, 0.3 equiv.) was co-distilled with dry toluene (three times, each time close to dryness) and then dissolved in dry toluene (3.5 mL). Silver(II) carbonate (1.45 g, 5.24 mmol, 1.5 equiv.) and subsequently the silver perchlorate/toluene solution were added to the suspension. Stirring was continued for 30 min in the cold before adding galactopyranosyl bromide **3** (2.07 g, 5.24 mmol, 1.5 equiv.), which was prepared according to the reported procedure,[5] in dry dichloromethane (13.0 mL) dropwise. The reaction mixture was allowed to warm up to room temperature and was stirred overnight. Additional bromide **3** (275 mg, 0.70 mmol, 0.2 equiv.) dissolved in dry dichloromethane (3.0 mL) and added dropwise. Stirring was continued overnight again. Completion of the reaction was followed by TLC (R_f = 0.14, CH/EtOAc 4:1). The solution was filtered over celite with subsequent washing of the Celite® with dichloromethane. The filtrate was washed twice with sat. sodium bicarbonate and once with brine, dried over sodium sulfate and the solvent was removed in vacuo. The residue was purified by column chromatography on silica (CH/EtOAc 3:1→2:1) to obtain a colorless solid (1.66 g, 2.20 mmol) which was directly subjected to acetylation. The compound was dissolved in pyridine (4.3 mL) thioacetic acid (4.3 mL) was added. The resulting solution was stirred for 72 h at room temperature. Afterwards, the solvent was removed by co-distillation with toluene. The crude was purified by column chromatography on silica (CH/EtOAc 2:1→1:2) to obtain glycoside

4 as a colorless solid (1.18 g, 1.53 mmol, 44%). R_f = 0.36 (CH/EtOAc 1:2). $[\alpha]_D^{20}$ = +58.79 (c = 10.50, $CHCl_3$). ^{1}H NMR (600 MHz, DMSO) δ 8.24 (d, $J_{NH,H2}$ = 8.2 Hz, 1H, N–H), 7.91–7.83 (m, 3H, N–H-; H-4-; H-5-Fmoc), 7.67–7.59 (m, 2H, H-1-; H-8-Fmoc), 7.45–7.37 (m, 2H, H-3-; H-6-Fmoc), 7.35–7.26 (m, 2H, H-2-; H-7-Fmoc), 7.22 (d, $J_{Tyr\text{-}o,Tyr\text{-}m}$ = 8.3 Hz, 2H, Tyr-$o_{a,b}$), 7.00 (d, $J_{Tyr\text{-}m,Tyr\text{-}o}$ = 8.5 Hz, 2H, Tyr-$m_{a,b}$), 5.91–5.77 (m, 1H, H-2-OAll), 5.49 (d, $J_{H1,H2}$ = 3.5 Hz, 1H, H-1), 5.38 (d, $J_{H4,H3}$ = 2.7 Hz, 1H, H-4), 5.30–5.15 (m, 3H, H-3, H-3-OAll), 4.59–4.53 (m, 2H, H-1-OAll), 4.42–4.34 (m, 1H, H-2), 4.29–4.20 (m, 4H, H-5; CH_2-Fmoc; Tyr$^{\alpha}$), 4.21–4.12 (m, 1H, H-9-Fmoc), 4.07–3.89 (m, 2H, H-$6_{a,b}$), 3.07–2.82 (m, 2H, Tyr$^{\beta}$), 2.37–1.81 (m, 12H, Ac–H). ^{13}C NMR (150.9 MHz, DMSO) δ 171.5, 170.0, 169.9, 169.8, 169.8 (C=O(COOAll); 4 Ac), 156.0, 156.0, 155.1 (C=O(Fmoc); Tyr-y; Tyr-p), 143.7, 143.7 (C-1_a-; C-8_a-Fmoc), 140.7, 140.7 (C-4_a-; C-5_a-Fmoc), 132.3 (C-2-OAll), 130.2, 130.2 (Tyr-$o_{a,b}$), 127.6, 127.6 (C-3-; C-6-Fmoc), 127.0, 127.0 (C-2-; C-7-Fmoc), 125.2, 125.2 (C-1-; C-8-Fmoc), 120.1, 120.1 (C-4-; C-5-Fmoc), 117.8 (C-3-OAll), 117.3, 117.3 (Tyr-$m_{a,b}$), 96.5 (C-1), 67.4 (C-3), 67.0, 67.0 (C-4; C-5), 65.7 (CH_2-Fmoc), 64.9 (C-1-OAll), 61.6 (C-6), 55.7 (Tyr$^{\alpha}$), 47.2 (C-2), 46.6 (C-9-Fmoc), 35.7 (Tyr$^{\beta}$), 22.4, 20.6, 20.4, 20.4 (4 Me(Ac)). HR-ESI-MS (pos), m/z: 795.2719 ([M + Na]$^+$, calc.: 795.2736), 790.3172 ([M + NH_4]$^+$, calc.: 790.3181), 773.2903 ([M + H]$^+$, calc.: 773.2916.

Compound 5: To a solution of compound **4** (970 mg, 1.26 mmol, 1.00 equiv.) in dry tetrahydrofuran (5 mL), phenylsilane (317 µL, 2.57 mmol, 2.05 equiv.) was added under inert atmophere. Then, tetrakis(triphenylphosphine)palladium(0) (73 µg, 0.06 mmol, 0.05 equiv.) was added and the reaction was stirred for 30 min at room temperature. Then, the solvent was removed *in vacuo* and the dark brown crude was purified by column chromatography on silica (EtOAc/MeOH 20:1→1:1). For further purification, it was passed over a C18-Cartridge (H_2O/MeCN 100:0→0:100) to give the carboxylic acid **5** (530 mg, 723 µmol, 58%). R_f = 0.69 (EtOAc/MeOH/AcOH/H_2O 60:3:3:2). $[\alpha]_D^{20}$ = +96.84 (c = 0.95, DMF). ^{1}H NMR (500 MHz, DMSO) δ 8.23 (d, $J_{NH,H2}$ = 8.1 Hz, 1H, N–H), 7.90–7.82 (m, 3H, NH-; H-4-; H-5-Fmoc), 7.67–7.59 (m, 2H, H-1-; H-8-Fmoc), 7.44–7.37 (m, 2H, H-3-; H-6-Fmoc), 7.36–7.23 (m, 2H, H-2-; H-7-Fmoc), 7.18 (d, $J_{Tyr\text{-}o,Tyr\text{-}m}$ = 8.3 Hz, 2H, Tyr-$o_{a,b}$), 6.96 (d, $J_{Tyr\text{-}m,Tyr\text{-}o}$ = 8.4 Hz, 2H, Tyr-$m_{a,b}$), 5.47 (d, $J_{H1,H2}$ = 3.3 Hz, 1H, H-1), 5.38 (d, $J_{H4,H3}$ = 3.4 Hz, 1H, H-4), 5.22 (dd, $J_{H3,H2}$ = 11.8, $J_{H3,H4}$ = 3.2 Hz, 1H, H-3), 4.43–4.35 (m, 1H, H-2), 4.30–4.21 (m, 2H, H-5; CH_{2a}-Fmoc), 4.20–4.11 (m, 2H, H-9-Fmoc; CH_{2b}-Fmoc), 4.10–4.02 (m, 1H, Tyr$^{\alpha}$), 4.00–3.89 (m, 2H, H-$6_{a,b}$), 3.13–3.04 (m, 1H, Tyr$^{\beta}{}_a$), 2.91–2.80 (m, 1H, Tyr$^{\beta}{}_b$), 2.55–1.75 (m, 12H, Me(Ac)). ^{13}C NMR (150.9 MHz, DMSO) δ 170.0, 169.9, 169.8, 169.8 (C=O(Ac)), 155.5, 155.5, 154.7 (C=O(Fmoc); Tyr-y; Tyr-p), 143.9, 143.9 (C-1_a-; C-8_a-Fmoc), 140.7, 140.7 (C-4_a-; C-5_a-Fmoc), 130.4, 130.3 (Tyr-$o_{a,b}$), 127.6, 127.6 (C-3-; C-6-Fmoc), 127.0, 127.0 (C-2-; C-7-Fmoc), 125.3, 125.2 (C-1-; C-8-Fmoc), 120.0, 120.0 (C-4-; C-5-Fmoc), 117.0, 117.0 (Tyr-$m_{a,b}$), 96.6 (C-1), 67.4 (C-3), 67.0, 67.0 (C-4; C-5), 65.3 (CH_2-Fmoc), 61.6 (C-6), 56.7 (Tyr$^{\alpha}$), 47.2 (C-2), 46.7 (C-9-Fmoc), 39.8 (Tyr$^{\beta}$), 22.4, 20.6, 20.4, 20.4 (4 Me(Ac)). HR-ESI-MS (pos), m/z: 1487.4845 ([2 M + Na]$^+$, calc.: 1487.4952), 1465.5035 ([2 M + H]$^+$, calc.: 1465.5133), 755.2247 ([M + Na]$^+$, calc.: 755.2422), 733.2523 ([M + H]$^+$, calc.: 733.2603).

Synthesis of the β-GlcNAc-Tyr amino acid building block:

Compound 7: A solution of Fmoc-tyrosine allyl ester **2** (3.0 g, 6.76 mmol, 1.0 equiv.) and trichloroacetimidate 6 (5.5 g, 8.8 mmol, 1.3 equiv.), which was prepared according to reported procedures, were added to a dry flask containing molecular sieve (4.2 g, 4 Å) and dissolved in dry dichloromethane (85.0 mL) under inert atmosphere. The mixture was stirred for 25 min at room temperature and was the cooled in an ice-bath. TMSOTf (245 µL, 2.0 mmol, 0.2 equiv.) was added dropwise and the reaction was stirred overnight, while the temperature was allowed to reach room temperature. The organic phase was washed twice with sat. sodium bicarbonate solution, once with brine and dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was purified by column chromatography on silica (CH/EtOAc 3:1→2:1) to give of glycoside **7** (2.8 g, 3.1 mmol, 46%). R_f = 0.33 (CH/EtOAc 2:1). $[\alpha]_D^{20}$ = +4.64 (c = 8.70, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.83–7.77 (m, 2H, H-4-; H-5-Fmoc), 7.60–7.54 (m, 2H, H-1-; H-8-Fmoc), 7.48–7.38 (m, 2H, H-3-; H-6-Fmoc), 7.35–7.30 (m, 2H, H-2-; H-7-Fmoc), 7.08–6.86 (m, 4H, Tyr-$o_{a,b}$; Tyr-$m_{a,b}$), 5.95–5.81 (m, 1H, H-2-OAll), 5.41–5.24 (m, 3H, H-$3_{a,b}$-OAll; N-H-Fmoc), 5.24–5.15 (m, 1H, H-4), 5.10 (d, $J_{NH,H2}$ = 9.2 Hz, 1H, N–H), 5.07–5.02 (m, 1H, H-3), 4.79–4.67 (m, 4H, H-1; Tyr$^{\alpha}$; CH_2-Troc), 4.63 (d, $J_{H1OAll,H2OAll}$ = 6.0 Hz, 2H, H-1-OAll), 4.54–4.46 (m, 1H, CH_{2a}-Fmoc), 4.23–4.13 (m, 2H, H-6_a; CH_{2b}-Fmoc), 4.12–4.00 (m, 2H, H-9-Fmoc; H-6_b), 3.89–3.81 (m, 1H, H-2), 3.52–3.42 (m, 1H, H-5), 3.21–2.95 (m, 2H, Tyr$^{\beta}$), 2.07–2.02 (m, 9H, Me(Ac)). ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 171.5, 171.3, 170.7, 169.5 (3 C=O(Ac); 1 C=O(OAll), 156.3, 156.3 (Tyr-*y*; Tyr-*p*), 155.6 (C=O(Fmoc)), 154.2 (C=O(Troc)), 144.1, 143.6 (C-1_a-; C-8_a-Fmoc), 141.3, 141.3 (C-4_a-; C-5_a-Fmoc), 131.4 (C-2-OAll), 130.6, 130.6 (Tyr-$o_{a,b}$), 128.0, 128.0 (C-3-; C-6-Fmoc), 127.3, 127.3 (C-2-; C-7-Fmoc), 125.6, 125.2 (C-1-; C-8-Fmoc), 120.3, 120.3 (C-4-; C-5-Fmoc), 117.8, 117.8 (Tyr-$m_{a,b}$), 99.8 (C-1), 95.6 (CCl_3-Troc), 74.6 (CH_2-Troc), 72.0, 71.8 (C-3-OAll; C-4), 68.5 (C-3), 67.1 (CH_2-Fmoc), 66.3 (C-1-OAll), 62.0 (C-6), 56.1 (C-2), 54.7 (Tyr$^{\alpha}$), 47.3 (C-9-Fmoc), 37.8 (Tyr$^{\beta}$), 20.8, 20.8, 20.7 (CH_3-Ac). HR-ESI-MS (pos), *m/z:* 929.1634 ([M + Na]$^+$, calc.: 929.1643), 922.2107 ([M + NH_4]$^+$, calc.: 922.2073), 905.1840 ([M + H]$^+$, calc.: 905.1853).

Compound 8: Compound **7** (2.01 g, 2.2 mmol, 1.0 equiv.) was dissolved in dry acetic acid/tetrahydrofuran (1:1, 40.0 mL) and zinc dust (2.90 g, 44.4 mmol, 20.0 equiv.) was added. The mixture was stirred overnight at 50 °C. Afterwards, the zinc was filtered off over a POR_3-filter and the filtrate was concentrated *in vacuo.* The residue was dissolved in acetic anhydride/pyridine (1:2, 20.0 mL), 4-dimethylaminopyridine (26.9 mg, 0.2 mmol, 0.1 equiv.) was added and the solution was stirred overnight at room temperature. The solvent was evaporated *in vacuo*, followed by co-distillation of the residue with toluene. The residue was purified by column chromatography on silica (CH/EtOAc 1:2) to give acetamide **8** as a colorless solid (1.67 g, 2.2 mmol, 97%). R_f = 0.45 (CH/EtOAc 1:4). $[\alpha]_D^{20}$ = +39.05 (c = 0.70, DMF). ^{1}H NMR (600 MHz, DMSO) δ 8.05 (d, $J_{NH,H2}$ = 6.8 Hz, 1H, N–H), 7.93–7.84 (m, 2H, H-4-; H-5-Fmoc), 7.68–7.62 (m, 2H, H-1-; H-8-Fmoc), 7.44–7.37 (m, 2H, H-3-; H-6-Fmoc), 7.35–7.27 (m, 2H, H-2-; H-7-Fmoc), 7.24–6.88 (m, 2H, Tyr-$m_{a,b}$; Tyr-$o_{a,b}$), 5.93–5.79 (m, 1H, H-2-OAll), 5.29 (d, $J_{H1,H2}$ = 12.8 Hz, 1H), 5.32–5.26 (m, 2H, H-1; H-3_a-OAll), 5.24–5.17 (m, 2H, H-4; H-3_b-OAll), 4.98–4.87 (m, 1H, H-3), 4.61–4.52 (m, 2H, H-1-OAll), 4.35–4.14 (m, 5H, CH_2-Fmoc; Tyr$^{\alpha}$; H-6_a; H-9-Fmoc), 4.13–3.94 (m, 3H, H-5; H-6_b; H-2), 3.06–2.81 (m, 2H, Tyr$^{\beta}$), 2.07–1.69 (m, 12H, 4 Me(Ac)). ^{13}C NMR (150.9 MHz, DMSO) δ 171.5 (C=O(OAll)), 170.0, 170.0, 169.4, 169.3 (C=O(Ac)), 155.9 (C=O(Fmoc)), 155.5 (Tyr-*y*; Tyr-*p*), 143.7, 143.7 (C-1_a-Fmoc; C-8_a-Fmoc), 140.7, 140.7 (C-4_a-; C-5_a-Fmoc), 132.3 (C-2-OAll), 130.2, 130.2 (Tyr-$o_{a,b}$), 127.6, 127.6 (C-3-; C-6-Fmoc), 127.1, 127.1 (C-2-; C-7-Fmoc), 125.2, 125.2 (C-1-; C-8-Fmoc), 120.1, 120.1 (C-4-; C-5-Fmoc), 117.8 (C-3-OAll), 116.3 (Tyr-$m_{a,b}$), 97.9 (C-1), 72.4 (C-4), 70.8 (C-5), 68.4 (C-3), 65.7 (CH_2-Fmoc), 64.9 (C-1-OAll), 61.7 (C-6), 55.8 (Tyr$^{\alpha}$), 53.2 (C-2), 46.6 (C-9-Fmoc), 35.6 (Tyr$^{\beta}$), 22.7, 20.5, 20.4, 20.4 (4 CH_3-Ac). HR-ESI-MS (pos), *m/z:* 795.2716 ([M + Na]$^+$, calc.: 795.2736), 773.2902 ([M + H]$^+$, calc.: 773.2916), 790.3169 ([M + NH_4]$^+$, calc.: 790.3181).

Compound 9: Allyl ester **8** (527 mg, 682 µmol, 1.0 equiv.) and phenylsilane (172 µL, 1.398 mmol, 2.05 equiv.) were added to a dry flask and dissolved in dry tetrahydrofuran/dimethylformamide (1:1, 4.0 mL) under inert atmosphere. Tetrakis(triphenylphosphine)palladium(0) (39 mg, 34 µmol, 0.05 equiv.) dissolved in dry tetrahydrofuran (2.0 mL) was added dropwise and the reaction was stirred for 1 h at room temperature. The solvent was removed *in vacuo* and the residue was purified by column chromatography

on silica (EtOAc/MeOH 10:1→1:1) and subsequent C18-cartridge (MeCN in H_2O, 40%→60%) to give the carboxylic acid **9** as a colorless solid (404 mg, 551 μmol, 81%). R_f = 0.81 (EtOAc/MeOH/H_2O/AcOH 60:3:3:2) $[\alpha]_D^{20} = +3.94$ (c = 1.10, DMF). ^{1}H NMR (600 MHz, DMSO) δ 8.05 (d, $J_{NH,H2}$ = 9.1 Hz, 1H, N–H), 7.88 (d, $J_{H4,H3} = J_{H5,H4}$ = 7.5 Hz, 2H, H-4-; H-5-Fmoc), 7.73 (d, $J_{NH,CH2\text{-}Fmoc}$ = 8.4 Hz, 2H, CH_2-Fmoc), 7.68–7.64 (m, 2H, H-3-; H-6-Fmoc), 7.44–7.38 (m, 2H, H-2-; H-7-Fmoc), 7.22 (d, $J_{Yo,Ym}$ = 8.5 Hz, 2H, Tyr-$o_{a,b}$), 6.92 (d, $J_{Ym,Yo}$ = 8.6 Hz, 2H, Tyr-$m_{a,b}$), 5.28 (d, $J_{H1,H2}$ = 8.5 Hz, 1H, H-1), 5.20 (t, J = 9.9 Hz, 1H, H-3), 4.91 (t, J = 9.8 Hz, 1H, H-4), 4.25–4.16 (m, 4H, H-$6_{a,b}$; CH_{2a}-Fmoc; H-9-Fmoc), 4.14–4.09 (m, 1H, Tyr$^{\alpha}$), 4.09–4.04 (m, 1H, H-5), 4.04–3.95 (m, 1H, H-2), 3.05–2.79 (m, 2H, Tyr$^{\beta}$), 2.02–1.92 (m, 12H, Me(Ac)). ^{13}C NMR (150.9 MHz, DMSO) δ 173.4, 169.7, 169.5, 169.3 (4 C=O(Ac); 1 COOH), 156.0 (C=O(Fmoc)), 155.5 (Tyr-γ; Tyr-p), 143.7 (C-1_a-; C-8_a-Fmoc), 140.7 (C-4_a-; C-5_a-Fmoc), 130.2 (Tyr-$o_{a,b}$), 127.6 (C-3-; C-6-Fmoc), 127.1 (C-2-; C-7-Fmoc), 125.3, 125.3 (C-1-; C-8-Fmoc), 120.1 (C-4-; C-5-Fmoc), 116.2 (Tyr-$m_{a,b}$), 97.8 (C-1), 72.5 (C-3), 70.8 (C-5), 68.4 (C-4), 65.6 (C-6), 61.6 (CH_2-Fmoc), 55.7 (Tyr$^{\alpha}$), 53.2 (C-2), 46.7 (C-9-Fmoc), 35.6 (Tyr$^{\beta}$), 20.5, 20.4, 20.4 (4 Me(Ac)). HR-ESI-MS (pos), *m/z:* 755.2405 ($[M+Na]^+$, calc.: 755.2423), 733.2590 ($[M+H]^+$, calc.: 733.2603), 750.2856 ($[M+NH_4]^+$, calc.: 750.2868).

Synthesis of the α-GlcNAc-Tyr amino acid building block

Compound 11: Fmoc-tyrosine-allyl ester **2** (2.2 g, 4.9 mmol, 1 equiv.) and compound 10 (3.5 g, 7.3 mmol, 1.5 equiv.), which was prepared according to reported procedures,[2b] were added to a dry flask containing molecular sieve (4.54 g, 4 Å) and dissolved in dry dichloromethane/diethyl ether (1:1, 55 mL) under inert atmosphere. The mixture was stirred for 1 h at room temperature and was then cooled to −20 °C. TMSOTf (265 μL, 1.5 mmol, 0.3 equiv.) in dry dichloromthane (5.0 mL) was slowly added and the reaction was stirred for 2.5 h in the cold. The mixture was then diluted with dichloromethane (30 mL) and quenched by the addition of sat. $NaHCO_3$ while keeping the temperature at −20 °C. The organic phase was then washed twice with sat. $NaHCO_3$, dried over Na_2SO_4, and purified by column chromatography on silica (5:1 CH / EtOAc) to give compound **11** (1.82 g, 2.4 mmol, 49%). 1H-NMR (600 MHz, $CDCl_3$), *δ (ppm)*: 7.77 (d, J = 7.5 Hz, 2H, H-4-; H-5-Fmoc), 7.79–7.74 (m, 2H, H-1-; H-8-Fmoc), 7.43–7.37 (m, 2H, H-3-; H-6-Fmoc), 7.34–7.28 (m, 2H, H-4-; H-7-Fmoc), 7.02 (s, 4H, Tyr-$m_{a,b}$; Tyr-$o_{a,b}$), 6.29 (d, J = 3.7 Hz, H-1), 5.93–5.84 (m, 1H, H-2-OAll), 5.45 (dd, J = 10.4, 9.5 Hz, 1H, H-3), 5.35–5.21 (m, 2H, H-1-OAll), 5.17–5.08 (m, 2H, H-4; N–H), 4.66 (dd, J = 13.8, 5.8 Hz, 1H, Tyr$^{\alpha}$), 4.62 (d, J = 5.7 Hz, 2H, H-3-OAll), 4.64–4.60 (m, 1H, CH_{2a}-Fmoc), 4.38–4.32 (m, CH_{2b}-Fmoc), 4.31–4.25 (m, 1H, H-6_a), 4.22–4.17 (m, 1H, H-9-Fmoc), 4.13–3.96 (m, 2H, H-6_b, H-5), 3.66 (dd, J = 10.5, 3.7 Hz, 1H, H-2), 3.15–3.03 (m, 2H, Tyr$^{\beta}$), 2.23–1.93 (m, 9H, Ac–H). 13 C-NMR (150.9 MHz, DMSO), *δ (ppm)*: 171.16, 170.59, 170.20, 169.78 (C=O(COOAll), 3 Ac); 155.62, 155.29 (C=O(Fmoc); Tyr-γ; Tyr-; Tyr-*p*), 143.90, 143.80 (C-1_a-; C-8_a-Fmoc), 141.46, 141.43 (C-4_a-; C-5_a-Fmoc), 131.45 (C-2-OAll), 130.86, 130.75 (Tyr-$o_{a,b}$), 127.89 (C-3-; C-6-Fmoc), 127.20 (C-2-; C-7-Fmoc), 125.19, 125.10 (C-1-; C-8-Fmoc), 120.15, 120.13 (C-4-; C-5-Fmoc), 119.41 (C-1-OAll), 116.82, 116.50 (Tyr-$m_{a,b}$), 90.08 (C-1), 70.37 (C-3), 68.46, 67.99 (C-4; C-5), 66.98 (CH_2-Fmoc), 66.30 (C-3-OAll), 61.63 (C-6), 60.82 (C-2), 54.90 (Tyr$^{\alpha}$), 47.30 (C-9-Fmoc), 37.49 (Tyr$^{\beta}$), 20.85, 20.79, 20.73 (3× Me(Ac)). HR-ESI-MS (pos), m/z: 1535.5086 ($[2M+Na]^+$, calc.: 1535.5178), 779.2457 ($[M+Na]^+$, calc.: 779.2535).

Compound 12: To a solution of compound **11** (1.72 g, 2.27 mmol) in pyridine (4.5 mL), thioacetic acid (4.5 mL) was added and the solution was stirred overnight at room temperature. Afterwards, the solvent was removed *in vacuo* and the residue was co-distilled with toluene. The crude was purified by column chromatography on silica (CH/EtOAc 3:1→1:2) to give acetamide **12** as a colorless amorphous solid (1.25 g, 1.62 mmol, 70%). R_f = 0.16 (CH/EtOAc 1:1). $[\alpha]_D^{20} = +76.29$ (c = 12.50, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, $J_{H4,H3} = J_{H5,H6}$ = 7.5 Hz, 2H, H-4-; H-5-Fmoc), 7.58–7.52 (m, 2H, H-1-; H-8-Fmoc), 7.42–7.36 (m, 2H, H-3-; H-6-Fmoc), 7.33–7.27 (m, 2H, H-2-; H-7-Fmoc), 7.05–6.95 (m, 4H, Tyr-$m_{a,b}$; Tyr-$o_{a,b}$), 5.90–5.83 (m, 2H, N–H, H-2-OAll), 5.50 (d, $J_{H1,H2}$ = 3.5 Hz, 1H, H-1), 5.41 (dd, J = 10.9, 9.4 Hz, 1H, H-3), 5.35 –5.23 (m, 2H, H-3-OAll), 5.21 (t, J = 9.8 Hz, 1H, H-4), 4.70–4.58 (m, 3H, Tyr$^{\alpha}$, H-1-OAll), 4.55–4.47 (m, 1H, H-2), 4.47–4.32 (m, 2H, $CH_{2a,b}$-Fmoc), 4.24–4.16 (m, 2H, H-6_a, H-9-Fmoc), 4.04–3.96 (m, 2H, H-6_b, H-5), 3.16–2.99 (m, 2H, Tyr$^{\beta}$), 2.10–1.90 (m, 12H, Me(Ac)). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 171.6, 171.2, 170.6, 170.2, 169.3 (4 C=O(Ac); 1 C=O (COOAll)), 155.6 (C=O(Fmoc)), 155.1, 155.1 (Tyr-*y*; Tyr-*p*), 143.9, 143.8 (C-1_a-; C-8_a-Fmoc), 141.4, 141.4 (C-4_a-; C-5_a-Fmoc), 131.4 (C-2-OAll), 130.8, 130.8 (Tyr-$o_{a,b}$), 127.9, 127.9 (C-3-; C-6-Fmoc), 127.1, 127.1 (C-2-; C-7-Fmoc), 125.1, 125.0 (C-1-; C-8-Fmoc), 120.1, 120.1 (C-4-; C-5-Fmoc), 119.3 (C-3-OAll), 116.5, 116.5 (Tyr-$m_{a,b}$), 95.8 (C-1), 71.2 (C-3), 68.6 (C-5), 68.0 (C-4), 67.0 (CH_2-Fmoc), 66.2 (C-1-OAll), 61.7 (C-6), 54.9 (Tyr$^{\alpha}$), 52.0 (C-2), 47.3 (C-9-Fmoc), 37.5 (Tyr$^{\beta}$), 23.2 (Me(NHAc)), 20.8, 20.7, 20.7 (Me(OAc)). HR-ESI-MS (pos), *m/z:* 795.2720 ($[M+Na]^+$, calc.: 795.2736).

Compound 13: To a solution of compound **12** (918 mg, 1.19 mmol, 1.00 equiv.) in dry tetrahydrofuran (4.5 mL), phenylsilane (300 μL) was added under inert atmosphere. Next, a solution tetrakis(triphenylphosphine)palladium(0) (69 mg, 0.06 mmol, 0.05 equiv.) in dry tetrahydrofuran (4.5 mL) was added and the reaction mixture was stirred at room temperature for 30 min. The n, the solvent was removed *in vacuo* and the residue was purified by passing over a C18-cartridge (H_2O/MeCN 40:60→60:40). After removal of the solvent *in* vacuo, the residue was further purified by column chromatography on silica (DCM/MeOH 1:0→1:1) and subsequent C18-cartridge (MeCN in H_2O, 40%→60%) to give the carboxylic acid **13** as a colorless solid (686 mg, 0.94 mmol, 79%). R_f = 0.85 (EtOAc/MeOH/AcOH/H_2O 60:3:3:2. $[\alpha]_D^{20} = +87.90$ (c = 8.40, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (d, $J_{H4,H3} = J_{H5,H6}$ = 7.7 Hz, 2H, H-4-; H-5-Fmoc), 7.59–7.51 (m, 2H, H-1-; H-8-Fmoc), 7.41–7.36 (m, 2H, H-3-; H-6-Fmoc), 7.32–7.27 (m, 2H, H-2-; H-7-Fmoc), 7.07–6.89 (m, 4H, Tyr-$o_{a,b}$; Tyr-$m_{a,b}$), 6.17 (d, $J_{NH,H2}$ = 9.3 Hz, 1H, N–H), 5.50 (d, $J_{H1,H2}$ = 1.8 Hz, 1H, H-1), 5.46–5.37 (m, 1H, H-3), 5.25–5.18 (m, 1H, H-4), 4.66–4.62 (m, 1H, Tyr$^{\alpha}$), 4.55–4.45 (m, 2H, H-2; CH_{2a}-Fmoc), 4.39–4.33 (m, 1H, CH_{2b}-Fmoc), 4.22–4.15 (m, 2H, H-9-Fmoc; H-6_a), 4.05–3.92 (m, 2H, H-6_b; H-5), 3.17–3.02 (m, 2H, Tyr$^{\beta}$), 2.12–1.88 (m, 12H, Ac–H). ^{13}C NMR (150.9 MHz) δ 174.0, 171.7, 171.3, 170.9, 169.5 (4 C=O(Ac); 1 C=O(COOH)), 155.8, 155.8 (Tyr-*y*; Tyr-*p*), 143.9, 143.8 (C-1_a-; C-8_a-Fmoc), 141.4, 141.4 (C-4_a-; C-5_a-Fmoc), 130.9, 130.9 (Tyr-$m_{a,b}$), 127.9, 127.9 (C-3-; C-6-Fmoc), 127.1, 127.1 (C-2-; C-7-Fmoc), 125.2, 125.1 (C-1-; C-8-Fmoc), 120.1, 120.1 (C-4-; C-5-Fmoc), 116.4, 116.4 (Tyr-$o_{a,b}$), 95.5 (C-1), 71.1 (C-3), 68.5 (C-5), 68.0 (C-4), 67.0 (CH_2-Fmoc), 61.8 (C-6), 54.7 (Tyr$^{\alpha}$), 52.1 (C-2), 47.3 (C-9-Fmoc), 37.1 (Tyr$^{\beta}$), 23.1, 20.9, 20.8, 20.7 (Me(Ac)). HR-ESI-MS (pos), *m/z:* 733.2592 ($[M+H]^+$, calc.: 733.2603), 755.2408 ($[M+Na]^+$, calc.: 755.2423), 750.2859 ($[M+NH_4]^+$, calc.: 750.2868).

General peptide synthesis methods: The peptides were synthesized on a 10 to 13 μmol scale by automated Fmoc-SPPS on a Syro I automated peptide synthesizer (Biotage) using TentaGel R resin preloaded with Fmoc-AA-Trt (Rapp Polymere, Tübingen). Standard Fmoc amino acids (8.0 equiv.) were automatically coupled for 40 min using HBTU/HOBt. The synthesis employed protected α-GlcNAc-, β-GlcNAc- and α-GalNAc serine, threonine and tyrosine amino acid building blocks, respectively. The glycosylated amino acids (1.5 equiv.) were pre-activated using HATU/HOAt and manually added to the resin and the coupling time was extended to 8 h. Fmoc was removed by treatment with 20% piperidine in DMF. The *N*-terminus of peptides for vaccine preparation were acetylated for two hours using acetic anhydride/HOBt/DIPEA in DMF. Peptides for microarray fabrication were modified with a triethyleneglycol spacer (TEG, 3 equiv.) was coupled to the *N*-terminus using HBTU/

HOBt/DIPEA in DMF for two hours, followed by Fmoc deprotection. Release of the glycopeptides from the resin with simultaneous removal of the amino acid side-chain protecting groups was performed using TFA/TIPS/H_2O (95:5:5). An *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine (Sp) was coupled to the *C*-terminus of the peptides for vaccine preparation using HATU/HOAt/DIPEA in DMF for four hours, and the Boc protecting group was removed by treatment with DCM/TFA (3:1) for four hours. All peptides were desalted on Sep-Pak Vac 6cc C18 cartidges (1 g, *Waters*) followed by removal of the *O*-acetyl protecting groups on the glycans either by treatment with catalytic amounts of NaOMe in MeOH at pH 9.0–9.5 or by treatment with 0.2 M NaOH in MeOH (pH 10.0). The desired peptides were obtained after purification by preparative HPLC.

General synthesis method of peptide vaccine conjugates: The antigen peptide was dissolved in EtOH/H_2O (1:1, 1 mL). Then, 3,4-diethoxycyclobut-3-en-1,2-dione (SQ, 1.1 equiv.) was added and the pH was adjusted to 8.0 with sat. aq. Na_2CO_3 over 5 min. The reaction was stirred for 3.5 h at room temperature and subsequently neutralized with AcOH. The solvent was removed under reduced pressure and the crude product was purified by HPLC. CRM (1.0 mg, 16.95 nmol) was dissolved in 100 μL 75 mM Na_2HPO_4 buffer (pH 9.0-9.5) and approximately 30 equiv. of the SQ-glycopeptide conjugates were added. The pH was adjusted to 9.5 with 5 mg/mL NaOH. The reaction was stirred at room temperature for 3 d. The vaccine construct was purified by ultrafiltration (14 000 g, 10 min, Amicon Ultra-0.5 mL 30 kDa cut-off).

General synthesis method of glycopeptide-BSA conjugates: The glycopeptide was dissolved in EtOH/H_2O (1:1, 1 mL). Then, 3,4-diethoxycyclobut-3-en-1,2-dione (1.1 equiv.) was added and the pH was adjusted to 8.0 with sat. aq. Na_2CO_3 over 5 min. The reaction was stirred for 3.5 h at room temperature and subsequently neutralized with AcOH. The solvent was removed under reduced pressure and the crude product was purified using C18 cartridges. Then, BSA (1 mg/100 μL) and 30 equiv. of glycopeptide-SQ were dissolved in 75 mM Na_2HPO_4 buffer (pH 9.0–9.5). The pH was adjusted to 9.5 with 5 mg/mL NaOH. The reaction was stirred at room temperature for 2 d. The BSA-conjugates were purified by ultrafiltration (14 000 g, 10 min, Amicon Ultra-0.5 mL 30 kDa cut-off, Merck Millipore).

Production of polyclonal rabbit antibodies: For antibody induction two rabbits were immunized with α-GalNAc-CRM vaccine-**1** (rabbits **R1** and **R2**) and β-GlcNAc-CRM vaccine-**2** (rabbits **R3** and **R4**), respectively, together with a lipopolysaccharide cocktail (LPS, Span 80, Tween 80, mineral oil and aqua bidest.). Three booster immunizations were carried out over 12 weeks (at days 7, 14 and 80). Sera were collected before immunization as negative control. Sera for immunological evaluation were collected at days 28 and 84.

General ELISA method: 96-well Nunc MaxiSorp flat bottom microtiter plates were coated with 50 μl/well of the respective BSA conjugates; or with BSA (negative control) (10 μg/mL in 0.1 M Na_2HPO_4 (pH 9.3)) overnight at 4 °C. The plates were washed three times with PBST (0.2% Tween-20) and subsequently blocked with 1%w/w BSA in PBST (0.05% Tween-20) for 1 h at 37 °C. The raised polyclonal antibodies (in PBST (0.05% Tween-20)/1%w/w BSA) were added to the wells (100 μL/well) in two-fold serial dilutions. The plates were incubated for 1 h at 37 °C and washed three times with PBST (0.2% Tween-20). Bound antibodies were detected using 50 μL/well of a secondary biotin XX F(ab')2 goat-anti-rabbit IgG (H + L) diluted 1:2000 in PBST (0.05% Tween-20)/1%w/w BSA for 1 h at 37 °C and the plates were washed three times with PBST (0.2% Tween-20). Next, 50 μL of a 1:2000 dilution of streptavidin-conjugated horseradish peroxidase in PBST (0.05% Tween-20)/1% w/w BSA were added to each well and incubation was continued for 1 h at 37 °C. After washing, the microtiter plates were developed in the dark at room temperature using 100 μL/well of a 1:1 solution of ABTS (1 mg/mL in citrate buffer (40 mM citric acid, 60 mM Na_2HPO_4))/30% H_2O_2 (diluted 1:4000 in citrate buffer). The absorbance of each well was measured at 410 nm with a Synergy H4 spectrophotometer (*BioTek*).

Microarray fabrication and binding studies: Glycopeptides were printed in 50 μM concentration in printing buffer (150 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 8.5) in replicates of seven (100 pL per feature) on NHS-activated hydrogel slides (Nexterion® slide H, Schott, Mainz, Germany) using a non-contact piezoelectric spotting device (iONE, M2 automation, Berlin, Germany) at a relative humidity of 50%. The unreacted NHS-groups were blocked by treatment with 25 mM ethanolamine in 100 mM sodium tetraborate buffer (pH 9.0) for 1 h at room temperature. The slides were incubated with a dilution series (100 μL/well) of polyclonal antibodies in PBST buffer (0.2% Tween-20) for 1 h at room temperature. The raised polyclonal antibodies were detected with a secondary biotinylated antibody (biotin XX F(ab')2 fragment of goat anti-rabbit igG (H + L), 2 mg/mL, Life Technologies) diluted at 1:1000 in PBST buffer (0.2% Tween-20) and incubated for 1 h at room temperature. The slides were subsequently incubated with Cy5-labeled streptavidin (1 mg/mL, Invitrogen) at 1:1000 dilution in PBST buffer (0.2% Tween-20) for 1 h at room temperature. Finally, the microarray slides were scanned at 635 nm using a microarray fluorescence reader (Typhoon Trio+ microarray scanner (Amersham) or *GenePix 4300 A* (Molecular Devices Corporation)). Reported signals represent the means of all spot replicates with standard deviations.

General SDS gel electrophoresis and western blot analysis: Proteins were diluted to 2 to 4 mg/mL with reducing Laemmli-loading buffer and boiled for 5 min at 95 °C. Samples (10 μL) were loaded onto a 10% polyacrylamide Tris-Glycine gel and size-fractionated by SDS gel electrophoresis (160 V, 1 h). The proteins were then transferred onto a PVDF membrane (0.2 μm) using a wet-transfer system. The membrane was washed three times with milliQ water and stained with Ponceau S. After washing three times for 5 min with TBST (20 mM Tris, 150 mM NaCl, 0.1% (v/v%) Tween-20, pH 7.5), the membrane was blocked with 5% milk protein in TBST for 1 h at room temperature. Then, the membrane was washed three times with TBST for 5 min. The membrane was probed with affinity purified rabbit antibodies **AP-1**, **AP-2**, and **AP-3** at dilutions of 1:2000 overnight at 4 °C, followed by washing for three times with TBST for 10 min. For detection, the membrane was incubated with a secondary Alexa Fluor 488 conjugated donkey anti-rabbit IgG at a dilution of 1:1000 for 1 h at room temperature and subsequently washed three times with TBST for 10 min. The blot was imaged using a ChemiDoc MP imaging system (BioRad).

α-GlcNAcylation of RhoA: RhoA-GST (100 μg, 2.06 nmol was incubated with UDP-*N*-acetylglucosamine (2 mM, 100 equiv.) and the glycosyltransferase domain of *Photorhabdus asymbiotica* protein toxin ($PaTox^G$, 2.68 μM) in 50 mM HEPES buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM $MnCl_2$ for 3 h at 30 °C. The total reaction volume was 100 μL. A sample of the reaction was analyzed by SDS-PAGE without further purification.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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[1] a) G. W. Hart, *Annu. Rev. Biochem.* **1997**, *66*, 315–335; b) F. I. Comer, G. W. Hart, *J. Biol. Chem.* **2000**, *275*, 29179–29182.
[2] a) D. J. Gill, H. Clausen, F. Bard, *Trends Cell Biol.* **2011**, *21*, 149–158; b) E. P. Bennett, U. Mandel, H. Clausen, T. A. Gerken, T. A. Fritz, L. A. Tabak, *Glycobiology* **2011**, *22*, 736–756.
[3] a) A. Halim, G. Brinkmalm, U. Ruetschi, A. Westman-Brinkmalm, E. Portelius, H. Zetterberg, K. Blennow, G. Larson, J. Nilsson, *Proc. Nat. Acad. Sci.* **2011**, *108*, 11848–11853; b) C. Steentoft, S. Y. Vakhrushev, M. B. Vester-Christensen, K. T. B. G. Schjoldager, Y. Kong, E. P. Bennett, U. Mandel, H. Wandall, S. B. Levery, H. Clausen, *Nat. Methods* **2011**, *8*, 977–982; c) J. C. Trinidad, R. Schoepfer, A. L. Burlingame, K. F. Medzihradszky, *Mol. Cell. Proteomics* **2013**, *12*, 3474–3488; d) S. Y. Vakhrushev, C. Steentoft, M. B. Vester-Christensen, E. P. Bennett, H. Clausen, S. B. Levery, *Mol. Cell. Proteomics* **2013**, *12*, 932–944; e) T. Jank, X. Bogdanovic, C. Wirth, E. Haaf, M. Spoerner, K. E. Bohmer, M. Steinemann, J. H. C. Orth, H. R. Kalbitzer, B. Warscheid, C. Hunte, K. Aktories, *Nat. Struct. Mol. Biol.* **2013**, *20*, 1273–1280; f) T. Jank, S. Eckerle, M. Steinemann, C. Trillhaase, M. Schimpl, S. Wiese, D. M. Van Aalten, W. Driever, K. Aktories, *Nat. Commun.* **2015**, *6*, 7807; g) D. Liu, Q. Wei, W. Xia, C. He, Q. Zhang, L. Huang, X. Wang, Y. Sun, Y. Ma, X. Zhang, Y. Wang, X. Shi, C. Liu, S. Dong, *J. Am. Chem. Soc.* **2021**, *143*, 20216–20223.
[4] J. Ma, T. Liu, A. C. Wei, P. Banerjee, B. O'Rourke, G. W. Hart, *J. Biol. Chem.* **2015**, *290*, 29141–29153.
[5] K. Aktories, *Nat. Rev. Microbiol.* **2011**, *9*, 487–498.
[6] a) J. Yu, M. Schorlemer, A. Gomez Toledo, C. Pett, C. Sihlbom, G. Larson, U. Westerlind, J. Nilsson, *Chem. Eur. J.* **2016**, *22*, 1114–1124; b) A. Halim, U. Westerlind, C. Pett, M. Schorlemer, U. Ruetschi, G. Brinkmalm, C. Sihlbom, J. Lengqvist, G. Larson, J. Nilsson, *J. Proteome Res.* **2014**, *13*, 6024–6032.
[7] R. Gibadullin, D. W. Farnsworth, J. J. Barchi Jr, J. C. Gildersleeve, *ACS Chem. Biol.* **2017**, *12*, 2172–2182.
[8] a) W. Koenigs, E. Knorr, *Chem. Ber.* **1901**, *34*, 957–981; b) H. Paulsen, J. P. Holck, *Carbohydr. Res.* **1982**, *109*, 89–107; c) U. Westerlind, A. Hobel, N. Gaidzik, E. Schmitt, H. Kunz, *Angew. Chem. Int. Ed.* **2008**, *47*, 7551–7556; *Angew. Chem.* **2008**, *120*, 7662–7667.
[9] T. Rosen, I. M. Lico, D. T. W. Chu, *J. Org. Chem.* **1988**, *53*, 1580–1582.
[10] P. Wang, J. Nilsson, G. Brinkmalm, G. Larson, X. F. Huang, *Chem. Commun.* **2014**, *50*, 15067–15070.
[11] a) R. R. Schmidt, J. Michel, *Tetrahedron Lett.* **1984**, *25*, 821–824; b) R. R. Schmidt, J. Michel, *Angew. Chem. Int. Ed.* **1980**, *19*, 731–732; *Angew. Chem.* **1980**, *92*, 763–764; c) R. R. Schmidt, *Angew. Chem. Int. Ed.* **1986**, *25*, 212–235; *Angew. Chem.* **1986**, *98*, 213–236.
[12] a) G. Giannini, R. Rappuoli, G. Ratti, *Nucleic Acids Res.* **1984**, *12*, 4063–4069; b) J. Yu, O. C. Grant, C. Pett, S. Strahl, R. J. Woods, U. Westerlind, *Chem. Eur. J.* **2017**, *23*, 3466–3473; c) U. Möginger, A. Resemann, C. E. Martin, S. Parameswarappa, S. Govindan, E.-C. Wamhoff, F. Broecker, D. Suckau, C. Lebev Pereira, C. Anish, P. H. Seeberger, D. Kolarich, *Sci. Rep.* **2016**, *6*, 20488–20502.
[13] a) S. Keil, C. Claus, W. Dippold, H. Kunz, *Angew. Chem. Int. Ed.* **2001**, *40*, 366–369; *Angew. Chem.* **2001**, *113*, 379–382; b) S. Dziadek, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem. Int. Ed.* **2005**, *44*, 7630–7635; *Angew. Chem.* **2005**, *117*, 7803–7808; c) U. Westerlind, H. Schroder, A. Hobel, N. Gaidzik, A. Kaiser, M. Niemeyer Christof, E. Schmitt, H. Waldmann, H. Kunz, *Angew. Chem. Int. Ed.* **2009**, *48*, 8263–8267; *Angew. Chem.* **2009**, *121*, 8413–8417.
[14] a) L. A. Carpino, A. El-Faham, C. A. Minor, F. Albericio, *Chem. Commun.* **1994**, 201–203; b) L. A. Carpino, *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398; c) V. Dourtoglou, B. Gross, V. Lambropoulou, C. Zioudrou, *Synthesis* **1984**, *1984*, 572–574.
[15] M. Trester-Zedlitz, K. Kamada, S. K. Burley, D. Fenyö, B. T. Chait, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 2416–2425.
[16] F. G. Hanisch *Biol. Chem.* **2001** *382*, 143–149.

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