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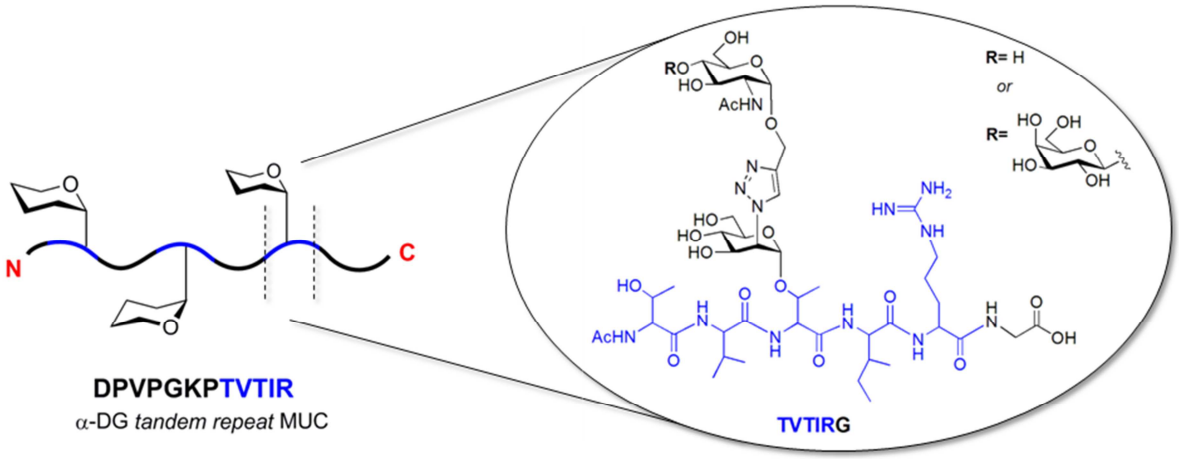

Synthesis of novel triazole-derived glycopeptides as analogs of α-dystroglycan mucins

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ABSTRACT

α-Dystroglycan (α-DG) mucins are essential for maintenance of the structural and functional stability of the muscle fiber and, when hypoglycosylated, they are directly involved in pathological processes such as dystroglycanopathies. Thus, this work reports the synthesis of the novel 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH (**1**) and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH (**2**), followed by solid-phase assembly to get the corresponding glycopeptides NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH (**3**) and NHAcThrVal[Gal-β1,4-αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH (**4**) as analogs of α-DG mucins. The glycosyl amino acids **1** (72%) and **2** (35%) were synthesized by Cu(I)-assisted 1,3-dipolar azide-alkyne cycloaddition reactions (CuAAC) between the azide-glycosyl amino acid αManN_3-FmocThrOBn (**5**) and the corresponding alkyne-functionalyzed sugars 2'-propynyl-αGlcNAc (**6**) and 2'-propynyl-Gal-β1,4-αGlcNAc (**7**), followed by hydrogenation reactions. Subsequently, glycopeptides **3** (23%) and **4** (12%) were obtained by solid phase synthesis, involving sequential couplings of Fmoc-protected amino acids or the glycosyl amino acids **1** and **2**, followed by cleavage from resin, *N*-acetylation and *O*-deacetylation (NaOMe) reactions. Lastly, enzymatic galactosylation of glycopeptide **3** with bovine β-1,4-GalT showed that it was not a substrate for this enzyme, which could be better elucidated by docking simulations with β-1,4-GalT.

Keywords: α-Dystroglycan; Mucin; Glycopeptide; Triazole; Synthesis; β-1,4-Galactosyltransferase

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1. Introduction

Dystroglycan (DG) is a core component of the dystrophin-glycoprotein (DGC) complex, which binds the cytoskeleton (actin) to extracellular matrix proteins (ECMs), via dystrophin and sarcoglycans, and is essential to maintain the structural and functional stability of the muscle fiber, enabling its successive cycles of contraction and relaxation, besides being expressed in other tissues such as epithelial and neural [1-3]. Dystroglycan (DG), encoded by the DAG1 gene, is biosynthesized as a precursor molecule that is further cleaved into α- and β-dystroglycan subunits (post-translational modification). β-Dystroglycan (β-DG) is a transmembrane protein that interacts with dystrophin through its intracellular domain, whereas α-dystroglycan (α-DG) is located outside the membrane and binds directly to extracellular matrix proteins including laminin, which is mostly characterized, agrin, perlecan and neurexin [4,5].

α-DG is a highly glycosylated protein composed by globular N- and C-terminal domains that are connected by a mucin-type domain, constituted by sequences rich in Ser/Thr, which represents potential sites for extensive *O*-glycosylation (Fig. 1A). Among the main peptide sequences identified in α-DG mucins is the DPVPGKPTVTIR sequence, whereas the major *O*-linked glycans found in α-DG mucins are represented by the tetrasaccharide structural motif Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-*O*-Ser/Thr, containing the peculiar O-mannose units (Fig. 1B), followed by the less expressed Galβ1-3GalNAcα-*O*-Ser/Thr, which is the most common *core* of other mammal mucins [4-7]. The α-DG tetrasaccharide biosynthesis is triggered by the action of distinct genes that encode the glycosyltransferases protein *O*-mannosyltransferases 1 and 2 (POMT1 and POMT2), and protein *O*-linked-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which catalyze the mannose and *N*-acetylglucosamine transfer reactions, respectively, as well as the LARGE glucosyltransferase and fukutin-related protein (FKRP), whose functions are not fully elucidated [7-9].

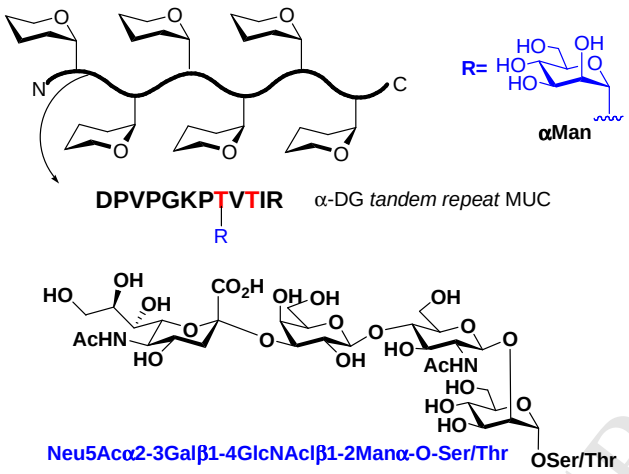


Figure 1. (A) General representation of α-DG mucins; **(B)** Chemical structure of the tetrasaccharide motif of α-DG.

Thus, abnormalities in the biosynthesis process of the above-mentioned *O*-mannosyl tetrasaccharide leads to hypoglycosylation of α-DG, disrupting its binding to the extracellular matrix, which in turn may result in various phenotypes of dystroglycanopathies known as congenital muscular dystrophies (CMDs), such as *Walker-Warburg* syndrome (WWS), *Muscle-eye-brain* disease (MEB) and *Limb-Girdle* muscular dystrophy (LGMD), which are manifested from the neonatal period and are generally characterized by progressive muscular degeneration, starting with muscle weakness and evolving to loss of movement and respiratory failure [7-12]. Despite their seriousness, so far there is no available treatment nor efficient diagnostic strategies directed to these dystroglycanopathies. Therefore, the synthesis of *O*-linked glycans found in α-DG mucins may represent a powerful tool to get synthetic substrates for glycosyltransferases involved in α-DG biosynthesis, which may guide the deeper investigation of their functions, favoring the development of effective therapeutic and diagnostic strategies towards CMDs.

The synthesis of α-DG tetrasaccharide *core* Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-*O*-Ser/Thr under different synthetic methods have been previously reported [13,14], as well as the synthesis of α-DG mucin glycopeptides bearing this tetrasaccharide *core* along with others containing the trisaccharide (Galβ1-4GlcNAcβ1-2Manα-*O*-Ser/Thr) and disaccharide (GlcNAcβ1-2Manα-*O*-Ser/Thr) intermediate *cores* [15,16]. In a distinct approach, a phospho-*O*-mannosyl trisaccharide glycopeptide derived from α-DG was also synthesized and applied for investigation of the enzyme factors that regulate *O*-glycans

extension in α-DG [17]. Within this context, in a continuous seek for novel α-DG-derived glycans, we asked whether or not the replacement of the β-1,2-glycosidic linkage between mannose and *N*-acetylglucosamine sugar units found in α-DG by *O*-triazole linkage could represent an efficient strategy to get peculiar α-DG mucins analogs as possible substrates for dystroglycan-related glycosyltransferases, as well as tools for the generation of antibodies against distinct glycoforms involved in the pathology of CMDs, which is an actual clinical demand. It is noteworthy the advantageous physicochemical properties of the triazole group, which is not susceptible to hydrolysis, oxidation or reduction, besides acting as a rigid link. Moreover, triazole derivatives can be promptly obtained by Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) reactions from suitable azide and alkyne functionalized precursors [18].

Thus, upon these considerations, here we report the synthesis of the novel 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH (**1**) and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH (**2**) (Fig. 2A), followed by solid-phase assembly to get the corresponding glycopeptides NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH (**3**) and NHAcThrVal[Gal-β1,4-αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH (**4**) (Fig. 2B) as analogs of α-DG mucins. Attempt for enzymatic galactosylation of glycopeptide **3** by bovine β-1,4-galactosyltransferase (β-1,4-GalT), as well as its docking simulations with β-1,4-GalT enzyme, will also be reported herein.

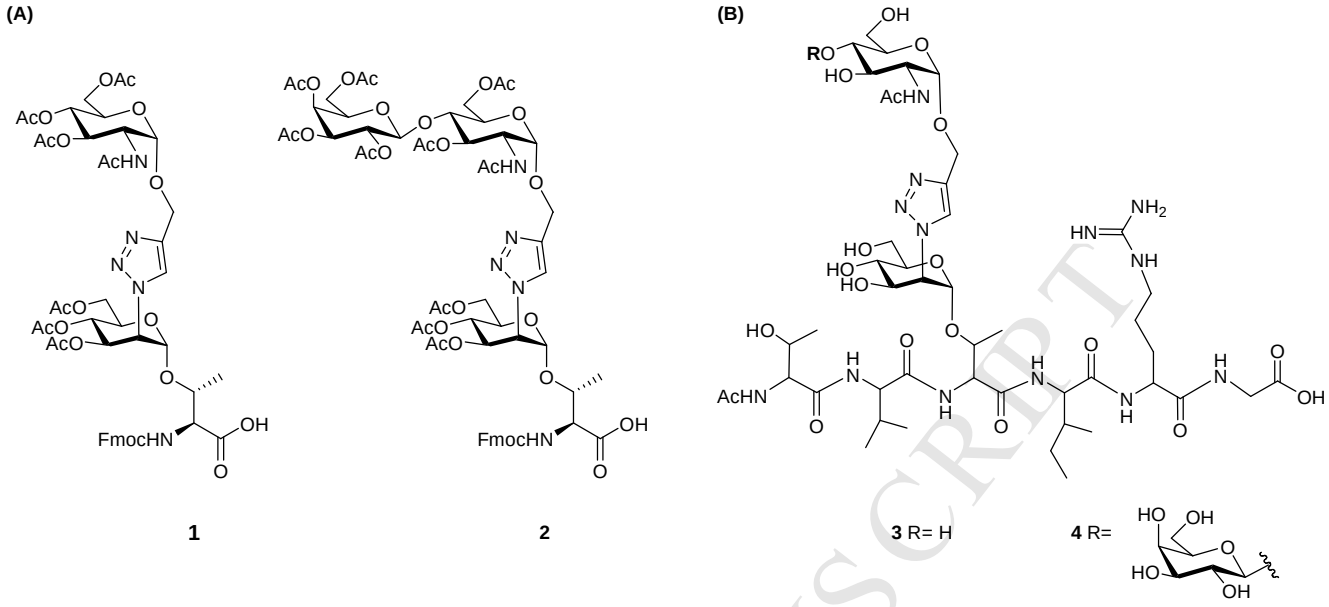


Figure 2. **(A)** Chemical structures of the 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2**. **(B)** Chemical structures of glycopeptides **3** and **4** as analogs of α-DG mucins.

2. Results and Discussion

2.1. Synthesis

The target glycopeptides NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH **3** and NHAcThrVal[Gal-β1,4-αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH **4** were obtained by solid-phase assembly, which required the prior synthesis of the 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2** as building blocks for obtaining α-DG mucins analogs.

2.1.1. Synthesis of 1,2,3-triazole-derived glycosyl amino acids

The chemical synthesis of the natural disaccharide (GlcNAcβ1-2Manα-O-Ser/Thr) and trisaccharide (Galβ1-4GlcNAcβ1-2Manα-O-Ser/Thr) cores of α-DG is not straightforward since it demands extensive synthetic routes involving previous selective functionalization reactions of donor and acceptor sugars, followed by laborious glycosylation reactions using specific catalysts under inert conditions. In this

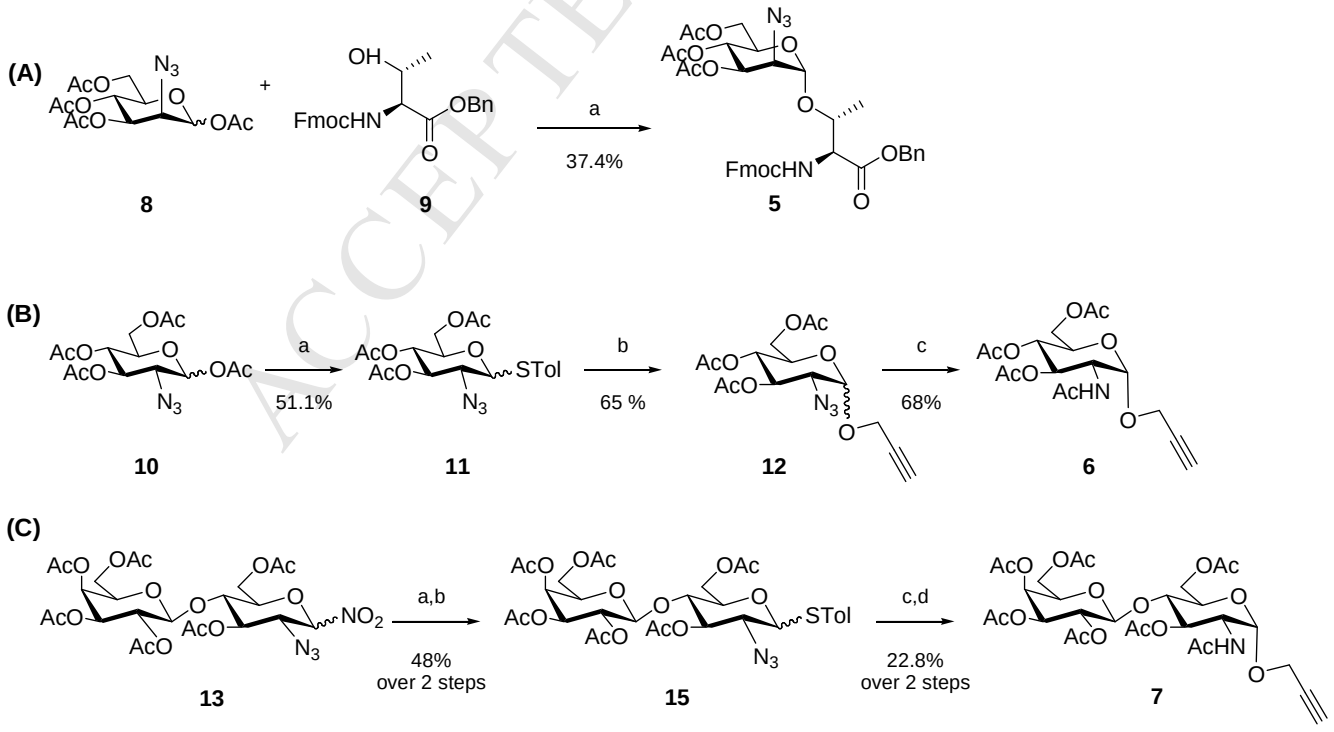
regard, the use of CuAAC reactions to obtain the novel 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2** as analogs of α-DG mucins may circumvent these drawbacks, considering the direct and selective functionalization of donor and acceptor sugars with alkyne and azide groups, which are prompt to the subsequent fast and highly selective CuAAC reactions to afford regioselective 1,4-disubstituted triazole in replacement to β-1,2-glycosidic linkage.

Therefore, to get the 1,2,3-triazole-derived glycosyl amino acids **1** and **2** it was necessary the previous synthesis of the azide-glycosyl amino acid αManN_3-FmocThrOBn (**5**) and the alkyne-based sugars 2'-propynyl-αGlcNAc (**6**) and 2'-propynyl-βGal-1,4-αGlcNAc (LacNAc) (**7**) as their direct precursors.

Firstly, the glycosylation reaction between the per-*O*-acetyl-2-azido-2-deoxy-mannosyl acetate (**8**) and the protected amino acid FmocThrOBn (**9**), utilizing $BF_3.Et_2O$ as catalyst and anhydrous dichloromethane as solvent,[19] afforded the azide-glycosyl amino acid αManN_3-FmocThrOBn **5** (40%) with α configuration after a 17 h reaction time (Scheme 1A). Compound **5** was characterized by IR spectra [2100 cm^{-1} (N_3)] and ^{1}H NMR analysis, which showed a characteristic doublet of sugar H-1 at δ 4.70 ($J_{1,2}$ 2,8 Hz), as well as amino acid related signals at δ 1.32 (CH_3-Thr) and δ 7.8-7.1 (*N*Fmoc and *O*Bn). HRESI-MS analysis of **5** showed the characteristic adduct 767.2536 $[M+Na]^+$.

Subsequently, the synthesis of the alkyne-based sugar 2'-propynyl-αGlcNAc **6** was initiated by glycosylation reaction of per-*O*-acetyl 2-azido-2-deoxy-glucosyl acetate (**10**) with p-thiocresol reagent, utilizing $BF_3.Et_2O$ as catalyst in anhydrous dichloromethane, giving the glycosyl donor GlcN_3STol (**11**) as an anomeric mixture (α:β 1:0.3, 51%) [20]. Due to difficulties to separate these anomers, this mixture of **11** was nextly employed to the glycosylation reaction with propargyl alcohol, in the presence of NIS/TMSOTf as a catalytic system in anhydrous dichloromethane, furnishing 2'-propynyl-GlcN_3 (**12**) as anomeric mixture (α:β 1:0.35, 65%) [21], which was submitted to the reductive acetylation of the azido group (zinc powder in THF/ acetic anhydride/ acetic acid) to give the final α anomer alkyne-functionalyzed 2'-propynyl-αGlcNAc **6** (68%) after chromatographic purification (Scheme 1B) [22]. The

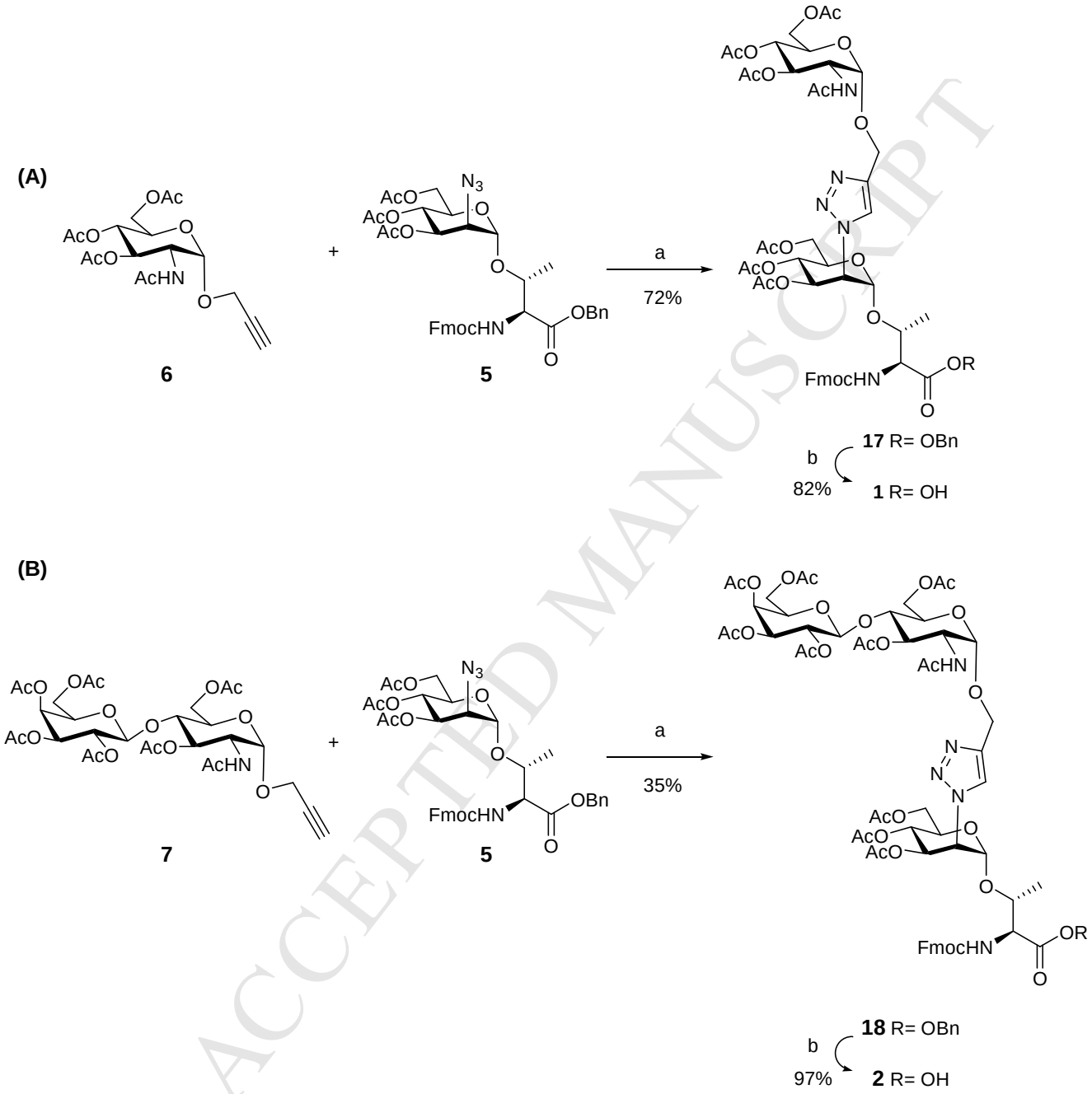
^{1}H NMR spectra of compound **6** showed characteristic signals of propargyl group (δ 2.52, *J* 2.5 Hz, CH) and NHAc (δ 5.8, *J* 9.0 Hz). HRESI-MS analysis of **6** showed the characteristic adduct 408.1268 $[M+Na]^+$. Regarding the preparation of the alkyne-based sugar 2'-propynyl-Gal-β1,4-αGlcNAc **7**, the first step involved the treatment of the lactose azido-nitrate (**13**) with NaOAc/ AcOH for conversion of the anomeric NO_2 to OAc, followed by glycosylation of the obtained per-*O*-acetyl 2-azido-2-deoxy-lactosyl acetate (**14**) (80%) with p-thiocresol reagent, under upon described conditions, to give the glycosyl donor $LacN_3STol$ (**15**) as an inseparable anomeric mixture (α:β 1:0.5, 60%) [20]. This mixture of **15** was then applied to the glycosylation reaction with propargyl alcohol (NIS/TMSOTf) to afford the 2'-propynyl-$LacN_3$ (**16**) as anomeric mixture (α:β 1:0.35, 65%) [21], which was treated with zinc powder and $CuSO_4$ (1M) in THF/ acetic anhydride/ acetic acid for reductive acetylation of the azido group, giving the final alkyne-functionalyzed 2'-propynyl-Gal-β1,4-αGlcNAc **7** (35%) (Scheme 1C) [22]. As described for **6**, the ^{1}H NMR spectra of compound **7** showed characteristic propargyl (δ 2.47, *J* 2.5 Hz, CH) and NHAc (δ 5.68, *J* 9.0 Hz) signals, whilst the HRESI-MS analysis of **7** showed the characteristic adduct 696.2116 $[M+Na]^+$.



Scheme 1. **(A)** Synthesis of azide-glycosyl amino acid αManN_3-FmocThrOBn **5**. Reagents and conditions: (a) $BF_3.Et_2O$, *p*-thiocresol, anhydrous DCM, RT, 24 h. **(B)** Synthesis of the alkyne-based sugar 2'-propynyl-αGlcNAc **6**. Reagents and conditions: (a) $BF_3.Et_2O$, *p*-thiocresol, anhydrous DCM, RT, 24 h; (b) Propargyl alcohol, NIS/ TMSOTf, anhydrous DCM, RT, 3 h; (c) Zn, $CuSO_4$, THF/Ac_2O/AcOH, 1 h. **(C)** Synthesis of the alkyne-based sugar 2'-propynyl-αLacNAc **7**; Reagents and conditions: (a) NaOAc/ AcOH, 100°C, 24 h; (b) $BF_3.Et_2O$, *p*-thiocresol, anhydrous DCM, RT, 24 h; (c) Propargyl alcohol, NIS/ TMSOTf, anhydrous DCM, RT, 3 h; (d) Zn, $CuSO_4$, THF/Ac_2O/AcOH, 1 h.

Once the azide-glycosyl amino acid αManN_3-FmocThrOBn **5** and the alkyne-functionalyzed sugars 2'-propynyl-αGlcNAc **6** and 2'-propynyl-Gal-β1,4-αGlcNAc **7** were obtained, the syntheses of protected 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOBn (**17**) and Gal-β1,4-αGlcNAc-1-*O*-triazol-2αMan-ThrOBn (**18**) were performed by Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition reactions (CuAAC), using the catalytic system $CuSO_4$/ sodium ascorbate and DMF as solvent, in a microwave reactor [18,23]. Thus, after 30 min. of microwave irradiation at 100 °C (150 W) the triazole-derived glycosyl amino acids (**17**) and (**18**) were obtained in corresponding yields of 72% and 35%, with regioselective formation of 1,4-disubstituted triazoles (Scheme 2). In order to improve the CuAAC reaction yield for the structurally more complex compound **18**, extended microwave irradiation time (60 min.) was applied, which did not lead, however, to increased yield, but only to degradation of starting materials. The ^{1}H NMR spectra of compounds **17** and **18** showed a characteristic CH-triazole singlet around δ 8.0, besides characteristic signals of $COCH_3$ groups (δ 2.2-1.8) and CH_3Thr (δ 1.36). HRESI-MS analyses of **17** and **18** showed the corresponding characteristic adducts of 1030.4066 $[M+H]^+$ and 1418.4845 $[M+H]^+$. The protected glycosyl amino acids **17** and **18** were then submitted to hydrogenation reactions (H_2/ Pd-C 10%) for removal of benzyl ester groups, affording the final αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** (82%) and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2** (97%) as prompt building blocks for solid-phase synthesis of glycopeptides **3** and **4** (Scheme 2). HRESI-

MS analyses of **1** and **2** showed the corresponding characteristic adducts of 1040.3441 $[M+H]^+$ and 1350.4281 $[M+Na]^+$.

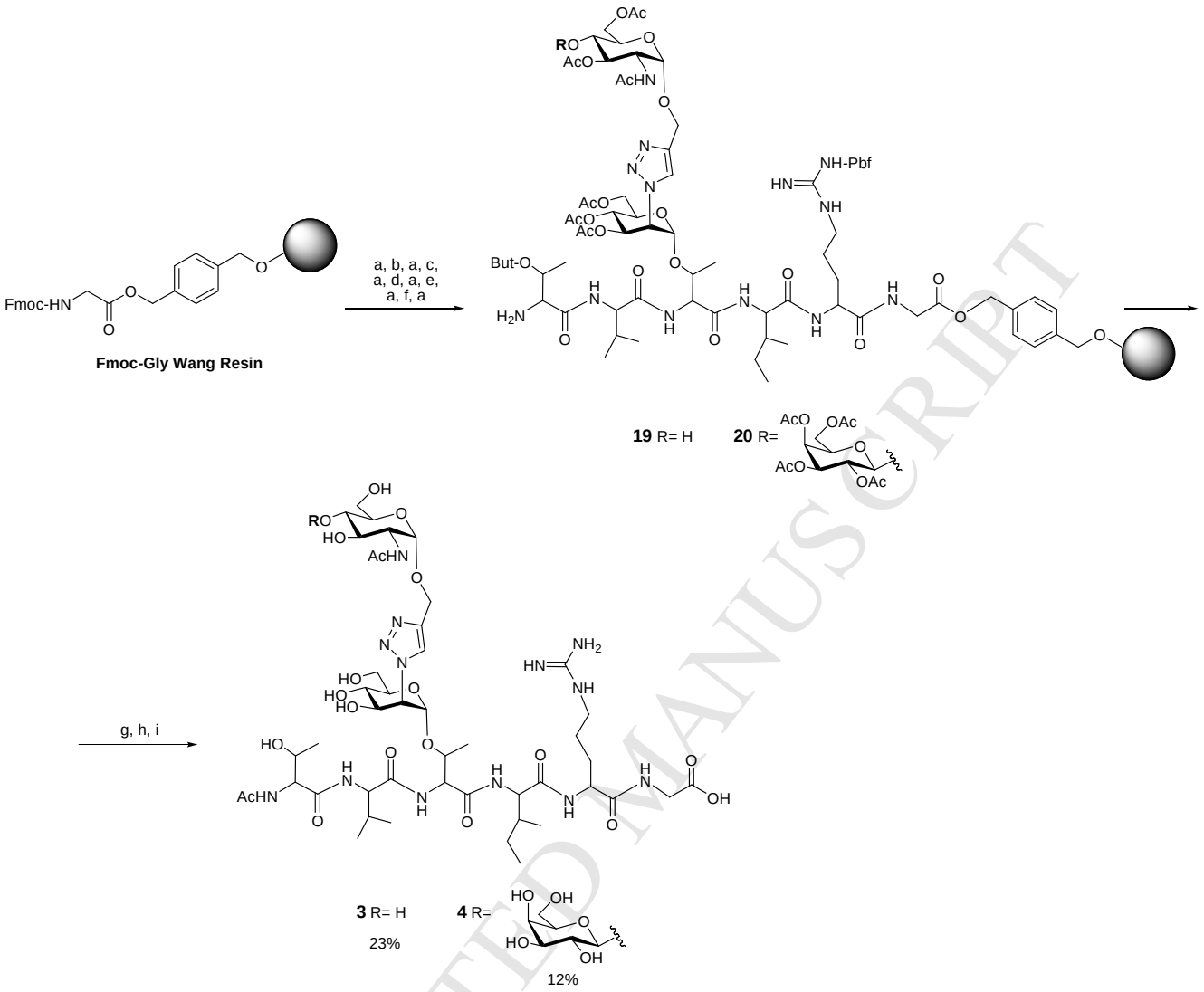


Scheme 2. **(A)** Synthesis of 1,2,3-triazole-derived glycosyl amino acid αGlcNAc-1-O-triazol-2αMan-ThrOH **1.** Reagents and conditions: (a) Na ascorbate, $CuSO_4$, DMF, microwave heating (100 °C, 150 W, 30 min); (b) H_2/Pd-C 10%, MeOH, AcOH, 0.5 h. **(B)** Synthesis of 1,2,3-triazole-derived glycosyl amino acid Gal-β1,4-αGlcNAc-1-O-triazol-2αMan-ThrOH **2**. Reagents and conditions: (a) Na ascorbate, $CuSO_4$, DMF, microwave heating (100 °C, 150 W, 30 min); (b) H_2/Pd-C 10%, MeOH, AcOH, 0.5 h.

2.1.2. Synthesis of glycopeptides

Moving towards more complex glycopeptides **3** and **4** as analogs of α-DG mucins, the orthogonal Fmoc-based solid-phase peptide synthesis (SPPS) was carried out, utilizing commercial Wang resin pre-loaded with Fmoc-Gly (0.65 mmol/g resin) as the solid support [22,24]. As the first step, *N*-Fmoc groups were removed with 50% morpholine in DMF, followed by coupling of the amino acids building blocks in the presence of the coupling reagents PyBOP/HOBt and *N*,*N*-diisopropylethylamine (DIPEA), in DMF.

Therefore, the synthesis of glycopeptides **3** and **4** comprised five rounds of coupling-deprotection with sequential incorporation of Fmoc-Arg(Pbf)OH, FmocIleOH, αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** or Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2**, FmocValOH and FmocThr(tBu)OH, affording the resin-bound protected glycopeptides (**19**) and (**20**) (Scheme 3). The obtained peptide coupling rates varied from 66% to 95%, as judged by spectrophotometrically detected dibenzofulvene released from the product [22, 24]. Subsequently, the obtained glycopeptides **19** and **20** were submitted to *N*-acetylation reactions (Ac_2O/ pyridine), followed by cleavage from resin in the presence of an aqueous mixture of trifluoracetic acid and triisopropylsilane (TFA:TIPS:H_2O, 90:5:5), with simultaneous removal of Pbf and OtBu protecting groups of the corresponding Arg and Thr amino acids. As the last step, removal of the sugar acetyl groups using NaOMe in MeOH furnished crude glycopeptides **3** and **4**, which were analysed and purified by C18 reverse phase column, being obtained in corresponding yields of 23% and 12%. The NMR ^{1}H spectra of glycopeptides **3** and **4** showed a characteristic C*H*-triazole singlet around δ 8.0, signals of sugars at δ 5.0 (H-1) and δ 1.9 (NH*Ac*), as well as methylene and methyl signals of amino acids between δ 3.12- δ 1.06. ESI-MS analyses also showed characteristic adducts for **3** ($[M + H]^+$ 1134.5639) and **4** ($[M + H]^+$: 1296.6166).

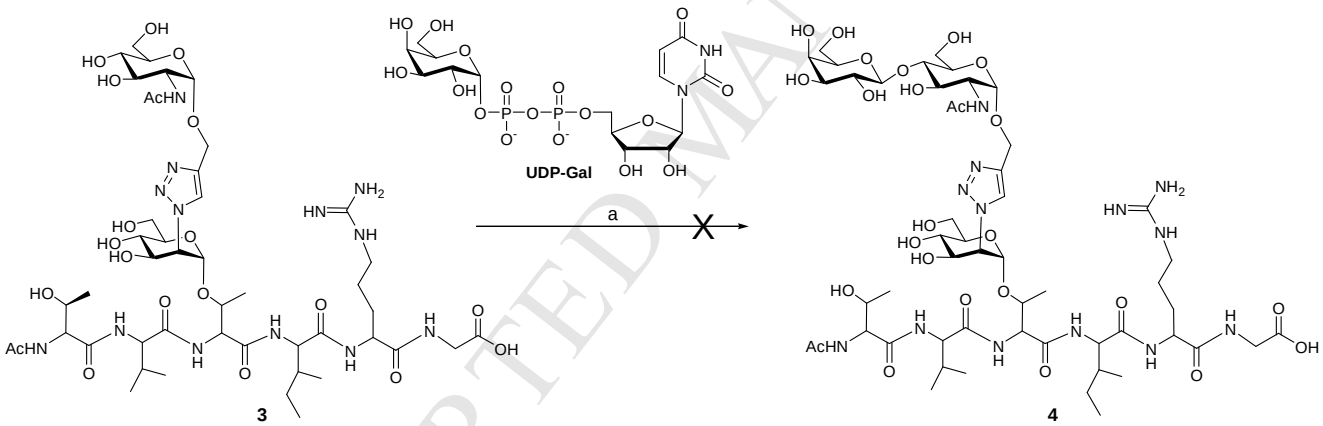


Scheme 3. Solid phase synthesis of glycopeptides **3** and **4**. Reagents and conditions: (a) 50% morpholine in DMF; (b) Fmoc-Arg(Pbf)OH, PyBOP, HOBt, DIPEA; (c) FmocIleOH, PyBOP, HOBt, DIPEA; (d) αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** or Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2**, PyBOP, HOBt, DIPEA; (e) FmocValOH, PyBOP, HOBt, DIPEA; (f) FmocThr(tBu)OH, PyBOP, HOBt, DIPEA; (g) Ac_2O/ pyridine; (h) TFA:TIPS:H_2O (90:5:5); (i) NaOMe/ MeOH.

2.1.3. Enzymatic galactosylation of glycopeptide 3

Despite the preference of the enzyme bovine β-1,4-galactosyltransferase (β-1,4-GalT) to galactosylate β-configured GlcNAc-acceptors, previous studies involving galactosylation of the α-configured αGlcNAc-ThrOH, in the presence of β-1,4-GalT, showed that it was possible to get the

desired galactosylated product αLacNAc-ThrOH at high enzyme concentration (2U/ mL) and longer reaction time (96 h) [24]. Therefore, it prompted us to investigate the capacity of glycopeptide **3** to act as substrate for bovine β1,4-GalT, despite bearing the unnatural α-linked glycosyl amino acid αGlcNAc-1-*O*-triazol-2Manα-ThrOH. Accordingly, galactosylation reaction of glycopeptide **3** was carried out utilizing the β-1,4-GalT enzyme, the donor substrate UDP-galactose (UDP-Gal) and $MnCl_2$ as cofactor, for 120 h at 37 °C (Scheme 4), and the obtained mixture was subsequently purified by gel filtration chromatography. However, the ESI-MS analysis of the isolated products did not confirm the structure of the expected galactosylated glycopeptide. This result led us to investigate the theoretical interactions between the glycopeptide **3** and β-1,4-GalT, which might have impaired the galactose transfer to **3**.



Scheme 4. Enzymatic galactosylation of glycopeptide **3**.

2.2. Molecular modeling studies

Docking simulations were performed using the crystallographic complex consisted of the bovine β-1,4-Galactosyltransferase 1 (β1,4GalT1) enzyme complexed with UDP-Gal and Mn^{2+} cofactor (PDB ID 1TVY) [25]. The docking protocol was validated by reproducing the experimentally observed position of Chitobiose, which is a known substrate for β1,4GalT1, at the acceptor site (PDB ID 1TW5) (Fig. S19). Both natural glycopeptide NHAcThrVal[GlcNAcβ1-2Manα]ThrIleArgGlyOH and its analog **3** were docked to the β1,4GalT1–Mn–UDPGal complex using this validated protocol, and their interaction modes were compared.

The results from docking simulations showed that the GlcNAc portion of both natural and triazole-glycopeptides can interact with the acceptor site of β1,4GalT1, in the same way as Chitobiose. According to the most likely complexes calculated by molecular docking, the GlcNAc portion of the natural glycopeptide makes hydrogen bonds with residues Tyr-289, Gly-316, Asp-318 and Arg-359. Additionally, there are hydrophobic interactions between the acetyl group of the sugar and the side chains of residues Phe-360 and Ile-363. The amino acid Trp-314 also composes the binding site and makes extensive CH-π interactions with the aliphatic ring of GlcNAc (Fig. 3A). This indicates that the GlcNAc portion of both natural substrate and triazole analog can be correctly recognized by the enzyme, and that the transfer reaction can proceed to generate the galactosylated product in both cases. In the case of the natural glycopeptide, our docking results show that its Man portion can make one hydrogen bond with residue Arg-359 (Fig. 3A). On the other hand, the Man portion of the triazole-disaccharide analog can make one hydrogen bond with Gln-288 and the triazole ring makes stacking interactions with Tyr-286 (Fig. 3B).

Although the correct orientation of the GlcNAc portion of the glycopeptide **3** indicate that it can act as acceptor substrate for β1,4GalT1, the galactosylated product was not detected in enzymatic reaction using this triazole-glycopeptide as substrate. The catalytic cycle of β1,4GalT1 involves conformational changes from an open and inactive state to a closed and active state. Such conformational change happens in the short and long loops, comprising residues 313-315 and 345-365, and is initiated by substrate binding to the acceptor site. The closed state is further stabilized by the binding of Mn^{2+} and UDP-Gal to the donor site. After catalysis, the enzyme must release the product and UDP in order to return to its open state and restart the catalytic cycle. In this context, it was observed that mutations that stabilize the closed state inhibit enzymatic activity due to the formation of a dead-end complex, which is locked in the closed state during the phase of product release [25]. Our docking calculations support the hypothesis that additional interactions established by the triazole moiety of glycopeptide **3**, specifically stacking with residue Tyr-286 (Fig. 3B), may stabilize the closed state of β1,4GalT1 and form a dead-end complex that effectively inhibits its enzymatic activity.

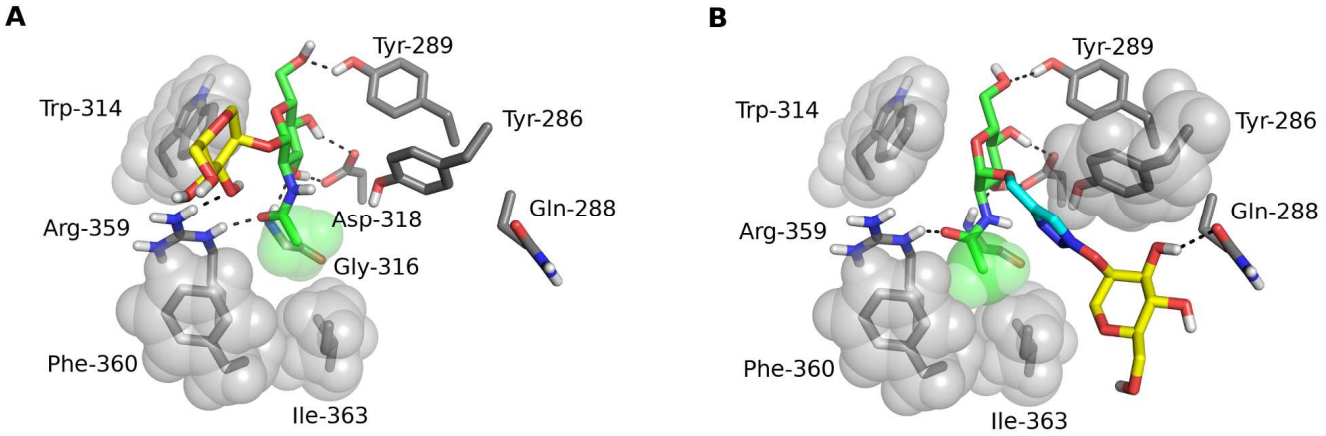


Figure 3. Interactions between the acceptor site of β1,4GalT1 and **A)** The natural α-DG glycopeptide NHAcThrVal[GlcNAcβ1-2Manα]ThrIleArgGlyOH or **B)** α-DG triazole glycopeptide NHAcThrVal[GlcNAcα1-*O*-triazol-2Manα]ThrIleArgGlyOH **3**, as calculated by molecular docking. Hydrogen, Nitrogen and Oxygen atoms are colored in white, blue and red, respectively. Carbon atoms are colored as follows: protein residues in gray, the GlcNAc portion of the disaccharides in green, the Man portion of the disaccharides in yellow and the CH_2-triazole moiety in cyan. Hydrogen bonds are indicated by dashed lines and hydrophobic interactions are indicated by transparent spheres.

For the sake of clarity, the peptide chains of both glycopeptides are not shown.

3. Conclusions

In summary, we obtained the novel 1,2,3-triazole-derived glycosyl amino acids αGlcNAc-1-*O*-triazol-2Manα-ThrOH **1** and Gal-β1,4-αGlcNAc-1-*O*-triazol-2Manα-ThrOH **2**, along with the corresponding glycopeptides NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH **3** and NHAcThrVal[Gal-β1,4-αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH **4** as analogs of α-DG mucins. In this regard, efficient synthetic routes led to the azide-glycosyl amino acid αManN_3-FmocThrOBn **5** and the alkyne-functionalyzed sugars 2'-propynyl-αGlcNAc **6** and 2'-propynyl-Gal-β1,4-αGlcNAc **7** as prompt building blocks for further CuAAC reactions, which proved to be effective to furnish the 1,2,3-triazole-derived glycosyl amino acids **1** and **2** with regioselective formation of 1,4-disubstituted triazoles.

Despite the feasibility of glycopeptide **3** to act as a substrate for bovine β1,4-GalT, the galactosylated glycopeptide **4** could not be obtained by enzymatic reaction with β1,4-GalT. This fact

could be elucidated by docking simulations with β-1,4-GalT, which showed that additional stacking interactions between the triazole moiety of glycopeptide **3** and the Tyr-286 residue might have interrupted the catalytic cycle of β-1,4-GalT, leading to its inhibition.

4. Experimental

4.1. General methods

All chemicals were purchased as reagent grade and used without further purification, unless otherwise noted. Reactions were monitored by thin layer chromatography (TLC) on precoated silica gel plates (Whatman, AL SIL G/UV, aluminium backing). Compounds were visualized under UV light (254 nm) and/or by dipping and heat with ethanol-sulfuric acid (95:5, v/v) visualization solution. Chromatographic purifications were carried out on Flash silica gel (40-63μm). Nuclear magnetic resonance spectra were recorded on Bruker Avance DRX 300 (300 MHz), DPX 400 (400 MHz) or DPX 500 (500 MHz) spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. Assignments were made with the aid of two-dimensional NMR spectra (COSY and HMQC) recorded on the same instruments. Mass spectrometric analyses were performed on a Bruker Daltonics MicroOTOF II ESI-TOF mass spectrometer, and accurate mass electrospray ionization mass spectra (ESI-HRMS) were obtained using positive or negative ionization modes.

4.2. Synthesis

4.2.1. *N*-(Fluoren-9-ylmethoxycarbonyl)-(2-azide-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannosyl)-L-threonine benzyl ester 5

Peracetylated mannosyl azide **8** (167.6 mg, 0.45 mmol, 1.0 equiv) and the previously synthesized FmocThrOBn **9** (96.6 mg, 0.22 mmol, 0.5 equiv) were solubilized in anhydrous dichloromethane (4.7 mL) and treated with $BF_3.Et_2O$ (110.8 μL, 0.90 mmol, 2.0 equiv). The mixture was stirred for 17 h and then concentrated under reduced pressure. The purification was performed by flash column chromatography (toluene/EtOAc 9:1, v/v) and the product **5** was obtained in 37.4% yield (125.1 mg, 0.168 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, 2H, *J* 7.2 Hz, C*H* Fmoc arom.), 7.68 (d, 2H, *J* 7.7

Hz, C*H* Fmoc arom.), 7.46-7.31 (m, 9 H, C*H* Fmoc arom, C*H* OBn arom), 5.67 (d, 1H, *J* 9.7 Hz, N*H* Thr), 5.32 (d, 2H, *J* 12.1 Hz, CH_2OBn,), 5.25 (d, 1H, $J_{3,4}$ 11.0 Hz, H-3), 5.15 (d, 1H, $J_{3,4}$ 11.0 Hz, H-4), 4.63 (broad s, 1H, H-1), 4.53 (dd, 1H, *J* 2.1 Hz, *J* 9.7 Hz, CHα-Thr), 4.50-4.44 (m, 2H, CH_2-Fmoc), 4.38-4.28 (m, 2H, C*H*-Fmoc, C*H*βThr), 4.20 (d, 1H, $J_{5,6a}$ 6.3 Hz, H-6a), 4.12-4.08 (m, 1H, H-6b), 3.98 (d, 1H, $J_{5,6a}$ 6.3 Hz, H-5), 3.44 (broad s, 1H, H-2), 2.14, 2.09, 2.08 (3 s, 9H, 3xCOCH_3), 1.37 (d, 3H, *J* 6.3 Hz, CH_3Thr). ^{13}C NMR (75 MHz. $CDCl_3$): δ 170.6 (*C*O Fmoc); 170.0; 169.8; 169.5 (*C*OCH_3), 156.6 (*C*O OBn), 143.7; 141.3; 137.8; 135.3 (*C* quat. Fmoc Ph), 135.1 (*C* quat.OCH_2Ph), 129.0; 128.9; 128.8; 128.6; 128.5; 128.2; 128,1, 127.8; 127.1; 125.3; 125.1; 120.0 (*C*H Ph), 99.3 (C-1), 70.7 (C-3), 69.1 (C-4), 67.6 (C-5), 67.2 (O*C*H_2 Fmoc), 66.2 (O*C*H_2-OBn), 62.3 (C-6), 61.3 (C-2), 58.6 (*C*HαThr), 47.2 (*C*H Fmoc), 21.4; 20.6; 20.5 (CO*C*H_3), 18.0 (*C*H_3Thr). ESI-HRMS: calcd. for $C_{38}H_{40}N_4NaO_{12}$ $[M + Na]^+$: 767.2535, found: 767.2536.

4.2.2. Tolyl 2-azido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-D-glucopyranoside 11

To a solution of peracetylated glucosyl azide **10** (300 mg, 0.83 mmol, 1.0 equiv) in anhydrous dichloromethane (4.0 mL) was added 4-methylbenzenethiol (*p*-thiocresol) (103.8 mg, 0.83 mmol, 1.0 equiv) and BF_3.Et_2O (309 μL; 2.5 mmol, 3.0 equiv). The mixture was stirred for 23 h at room temperature and followed by TLC. The reaction was then quenched with triethylamine, concentrated *in vacuo* and purified by silica gel column chromatography (gradient toluene 100% → toluene/EtOAc 8:2, v/v) to give the product **11** as an inseparable anomeric mixture (α:β 1:0.3) in 51.1% yield. ^{1}H NMR (300 MHz, $CDCl_3$), α anomer: δ 7.39 (d, 2H, J_{orto} 8.1 Hz, C*H* STol arom.), 7.24 (d, 2H, J_{orto} 8.1 Hz, C*H* STol arom.), 5.57 (d, 1H, $J_{1,2}$ 5.6 Hz, H-1), 5.37-5.34 (m, 1H, H-3), 5.10-5.00 (m, 1H, H-4), 4.62 (dd, 1H, $J_{5,6a}$ 5.0 Hz, $J_{5,6b}$ 10.2 Hz, H-5), 4.30 (d, 1H, $J_{5,6a}$ 5.1 Hz, H-6a), 4.09-4.01 (m, 2H, H-2, H-6b), 2.34 (s, 3H, CH_3-thiocresol), 2.10, 2.06, 2.05 (3 s, 12H, 3x CH_3CO). ^{13}C NMR (75 MHz. $CDCl_3$): δ 171.5, 170.6, 170.1, 169.5 (CH_3*C*O), 132.7 (*C*H STol), 129.9 (*C*H Stol), 86.8 (C-1), 71.3 (C-3), 68.6 (C-4), 68.3 (C-5), 62.0

(C-6), 61.7 (C-2), 20.9 (*C*H_3 STol), 20.4-18.8 (3x*C*H_3CO). ESI-HRMS: calcd. for $C_{19}H_{23}N_3NaO_7S$ [M + Na]$^+$: 460.4560, found: 460.1147.

4.2.3. 2'-propynyl-2-azide-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranoside 12

To a solution of glycosyl donor $GlcN_3STol$ **11** (84 mg, 0.19 mmol, 1.1 equiv) and N-iodosuccinimide (NIS) (9.0 mg, 0.26 mmol, 1.5 equiv) in anhydrous dichloromethane (2.5 mL) was added propargyl alcohol (30 μL, 0.52 mmol, 3.0 equiv). The mixture was then treated with TMSOTf (3.2 μL, 0.019 mmol, 0.1 equiv) and stirred for 3 h at room temperature. The resulting mixture was diluted with dichloromethane, washed with both saturated $NaHCO_3$ 10% and sodium thiosulphate solutions, dried over $MgSO_4$ and purified by silica gel column chromatography (gradient toluene 100% → toluene/EtOAc 8:2, v/v) to afford the product **12** in 65 % yield (46.9 mg, 0.124 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): α anomer: δ 5.50 (dd, 1H, $J_{3,4}$ 8.8 Hz, $J_{4,5}$ 10.6 Hz, H-4), 5.38 (d, 1H, $J_{3,4}$ 8.8 Hz, H-3), 5.23 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 4.34 (d, 2H, *J* 2.4 Hz, CH_2-prop.), 4.16-4.07 (m, 3H, H-5, H-6a, H-6b), 4.43 (dd, 1H, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 10.6 Hz, H-2), 2.52 (t, 1H, *J* 2.4 Hz, C*H*-prop.), 2.11, 2.10, 2.05 (3 s, 12H, 3x CH_3CO). ^{13}C NMR (75 MHz. $CDCl_3$): δ 171.7, 170.7, 170.0, 169.2 (CH_3*C*O), 96.3 (C-1), 71.1 (C-3), 78.2 (C prop.), 75.4 (*C*H prop.), 68.0 (C-4), 68.3 (C-5), 62.0 (C-6), 61.7 (C-2), 55.3 (CH_2 prop.), 23.1, 20.7, 20.5 (*C*H_3CO). ESI-HRMS: calcd. for $C_{15}H_{20}KN_3O_8$ [M + K]$^+$: 409.0876, found: 409.1624.

4.2.4. 2'-propynyl-2-acetamide-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside 6

To a solution of compound **12** (72.4 mg; 0.196 mmol, 1.0 equiv) in a mixture of THF/acetic anhydride/acetic acid 3:2:1 v/v/v (3.0 mL) was added zinc dust (77 mg, 1.18 mmol, 6.0 equiv) and 1M $CuSO_4$ solution (40 μL). After stirring for 2 hours at room temperature the reaction mixture was filtered through Celite and purified by silica gel column chromatography (gradient EtOAc/hexane 1:1→EtOAc/hexane 7:3, v/v), affording compound **6** (α anomer) in 68% yield (51.2 mg, 0.133 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.77 (d, 1H, $J_{2,NH}$ 8.9 Hz, NHAc), 5.26-5.20 (m, 1H, H-3), 5.38 (t, 1H, $J_{3,4} = J_{4,5}$ 9.6 Hz, H-4), 5.03 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 4.40 (td, 1H, $J_{1,2}$ 3.7 Hz, $J_{2,3} = J_{2,NHAc}$ 10.1 Hz, H-2),

4.27 (t, 2H, *J* 2.4 Hz, C*H*$_2$-prop.), 4.23 (d, 1H, $J_{5,6a}$ 4.2 Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b}$ 2.3 Hz, $J_{6a/6b}$ 12.3 Hz, H-6b), 4.00 (ddd, 1H, $J_{5,6b}$ 2.3 Hz, $J_{5,6a}$ 4.2 Hz, $J_{4,5}$ 9.7 Hz, H-5), 2.49 (t, 1H, *J* 2.4 Hz, C*H*-prop.) 2.10 (s, 3H, CH_3CONH), 2.03, 2.02, 1.97 (3 s, 12H, 3x C*H*$_3$CO). ^{13}C NMR (75 MHz. $CDCl_3$): δ 171.7, 170.7, 170.0, 169.2 (CH_3CO), 96.3 (C-1), 71.1 (C-3), 78.2 (C prop.), 75.4 (*C*H prop.), 68.0 (C-4), 68.3 (C-5), 62.0 (C-6), 55.3 (CH_2 prop.), 51.4 (C-2), 23.1 (*C*H_3CONH), 20.7-20.5 (3x*C*H_3CO). ESI-HRMS: calcd. for $C_{17}H_{23}NO_9Na$ $[M + Na]^+$: 408.1265, found: 408.1268.

4.2.5. Tolyl-3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-1-thio-D-glucopyranoside 15

To a solution of 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-1-thio-D-glucopyranosyl nitrate **13** (500 mg, 0.75 mmol, 1.0 equiv.) in acetic acid (17 mL) was added sodium acetate (225 mg, 2.74 mmol, 3.6 equiv) and the reaction mixture was stirred for 24 h at 100°C. The resulting mixture was then diluted with ethyl acetate, washed with saturated $NaHCO_3$, dried over $MgSO_4$ and purified by silica gel column chromatography (hexane/EtOAc, 3:7 v/v) to afford the compound 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-1-thio-D-glucopyranosyl acetate **14** (396.93 mg, 0.6 mmol, 80%) as an amorphous solid. To a solution of compound **14** (300 mg, 0.45 mmol) in anhydrous dichloromethane were added *p*-thiocresol (56 mg, 0.45 mmol, 1.0 equiv) and $BF_3.Et_2O$ (167.7 µL, 1.33 mmol, 3.0 equiv), and the reaction mixture was stirred for 12 h at room temperature. The reaction mixture was quenched with triethylamine and purified by silica gel column chromatography (EtOAc/Hexane 1:1 v/v) to give compound **15** as an inseparable anomeric mixture (α:β 1:0.5) in 60% yield (197.2 mg, 0.27 mmol). ^{1}H NMR (300 MHz, $CDCl_3$), α anomer: δ 7.38 (d, 2H, J_{ortho} 7.5 Hz, C*H* STol arom.), 7.13 (d, 2H, J_{ortho} 7.0 Hz, C*H* STol arom.), 6.26 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 5.38 (d, 1H, $J_{3',4'}$ 2.0 Hz, H-4'), 5.25-5.16 (m, 2H, H-2', H-3), 5.13 (dd, 1H, $J_{2',3'}$ 10.1 Hz, $J_{3',4'}$ 2.0 Hz, H-3'), 5.02-4.93 (m, 3H, H-6b, H-1', H-6a,), 4.90-4.84 (m, 1H, H-5'), 4.23-4.07 (m, 2H, H-6b', H-6a'), 3.74 (t, 1H, *J* 9.5 Hz, H-4), 3.65-3.58 (m, 1H, H-5), 3.48 (dd, 1H, $J_{1,2}$ 3.8 Hz, $J_{2,3}$ 10.5 Hz, H-2), 2.35 (s, 3H, CH_3-thiocresol), 2.19-2.02 (6s, 18H, 6xCOC*H*$_3$). ^{13}C NMR (75 MHz. $CDCl_3$): δ 169.3-167.8

(*C*OCH$_3$), 132.7 (*C*H STol), 129.7 (*C*H Stol), 101.2 (C-1'), 85.7 (C-1), 74.3 (C-4), 71.0 (C-3'), 70.7 (C-5'), 69.2 (C-3), 69.1 (C-2'), 66.8 (C-5), 66.6 (C-4'), 62.4 (C-6), 62.1 (C-6'), 60.8 (C-2), 29.7 (*C*H$_3$ STol), 21.1-20.5 (3x*C*H$_3$CO). ESI-HRMS: calcd. for $C_{31}H_{39}N_3NaO_{15}S^+$ $[M + Na]^+$: 748.1994, found: 748.1996.

4.2.6. 2'-propynyl-3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-α-D-glucopyranoside 16

The lactosyl donor **15** (127 mg, 0.175 mmol, 1.0 equiv.) and N-iodosuccinimide (NIS) (45.3 mg, 0.2 mmol, 1.15 equiv) were solubilized in anhydrous dichloromethane (3.5 mL), followed by addition of propargyl alcohol (28.3 µL, 0.52 mmol, 3.0 equiv). The mixture was then treated with TMSOTf (1.55 µL, 0.0175 mmol, 0.1 equiv) and stirred for 3 h at room temperature. The resulting mixture was diluted with dichloromethane, washed with both saturated $NaHCO_3$ 10% and sodium thiosulphate solutions, dried over $MgSO_4$ and purified by silica gel column chromatography (gradient toluene 100% → toluene/EtOAc 8:2, v/v) to afford the product **16** as an anomeric mixture (α:β 1:0.35) in 65% yield (46.9 mg, 0.124 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): α anomer: δ 6.19 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.35 (d, 1H, $J_{3',4'}$ 3.6 Hz, H-4'), 5.33-5.27 (m, 2H, H-2', H-3), 5.11 (dd, 1H, $J_{2',3'}$ 7.4 Hz, $J_{3',4'}$ 3.6 Hz, H-3'), 4.51 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1'), 4.19 (t, 2H, *J* 2.4 Hz, C*H*$_2$-prop.), 4.10-4.07 (m, 3H, H-5', H-6a', H-6b'), 4.04-4.03 (m, 3H, H-5, H-6a, H-6b), 3.93 (d, 1H, $J_{3,4}$ 9.7 Hz, H-4), 3.45 (dd, 1H, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 10.6 Hz, H-2), 2.47 (t, 1H, *J* 2.4 Hz, C*H*-prop.), 2.11-1.99 (6s, 18H, 6xCOC*H*$_3$). ^{13}C NMR (75 MHz. $CDCl_3$): δ 170.5-170.1 (*C*OCH$_3$), 101.3 (C-1'), 90.0 (C-1), 75.7 (C prop.), 75.6 (*C*H prop.), 74.1 (C-5'), 71.0 (C-3'), 70.7 (C-5), 70.2 (C-2'), 69.6 (C-3), 66.8 (C-4), 66.6 (C-4'), 62.1 (C-6'), 61.3 (C-6), 60.7 (C-2), 53.4 (CH_2 prop.), 21.0-20.5 (3x*C*H$_3$CO). ESI-HRMS: calcd. for $C_{27}H_{35}N_3NaO_{16}$ $[M + Na]^+$: 680.1910, found: 680.1906.

4.2.7. 2'-propynyl-3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranoside 7

To a solution of compound **16** (112 mg, 0.16 mmol, 1.0 equiv.) in a mixture of THF/acetic anhydride/acetic acid 3:2:1 v/v/v (3.0 mL) were added zinc dust (60 mg, 0.9 mmol; 6.0 equiv) and 1M aqueous $CuSO_4$ solution (30 µL), and the reaction mixture was stirred for 1 h at room temperature. The mixture was filtered through Celite and purified by silica gel column chromatography (gradient EtOAc/Hexane 7:3 v/v → EtOAc 100%) to afford compound **7** (α anomer) in 35% yield (37.4 mg, 0.055 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.62 (d, 1H, *J* 9.2 Hz, NH), 5.33-5.29 (m, 2H, H-4', H-3), 5.12 (dd, 1H, $J_{1',2'}$8.0 Hz, $J_{2',3'}$10.4 Hz, H-2'), 4.96 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.4 Hz, H-3'), 4.92 (broad s, 1H, H-1), 4.63-4.60 (m, 1H, H-2), 4.57 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1'), 4.31 (dd, 1H, $J_{5,6b}$ 1,8 Hz, $J_{6a,6b}$ 11.8 Hz, H-6b), 4.23 (dd, 1H, *J* 2.4 Hz, *J* 11.8 Hz, CH_2-prop.), 4.23-4.16 (m, 2H, H-4, H-6b), 4.01 (dd, 1H, $J_{5,6a}$ 7.7 Hz, $J_{6a,6b}$ 11.2 Hz, H-6a), 3.97-3.94 (m, 1H, H-6a'), 3.86 (t, 1H, $J_{5,6a/6b}$ 6.9 Hz, H-5), 3.76 (t, 1H, $J_{5',6a'/6b'}$ 9.6 Hz, H-5'), 2.45 (t, 1H, *J* 2.2 Hz, C*H*-prop.), 2.15 (s, 3H, CH_3CONH), 2.12-1.96 (18 H, 6 s, 6xCOCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5-169.3 (*C*OCH_3), 100.8 (C-1'), 96.0 (C-1), 78.0 (C prop), 75.7 (*C*H prop.), 74.4 (C-5'), 71.0 (C-3'), 70.6 (C-5), 69.9 (C-2'), 69.3 (C-3), 69.1 (C-4), 66.7 (C-4'), 62.7 (C-6'), 60.9 (C-6), 54.8 (CH_2 prop.), 49.9 (C-2), 23.4 (*C*H_3CONH), 21.0-20.5 (3x*C*H_3CO). ESI-HRMS: calcd. for $C_{29}H_{39}NNaO_{17}$ $[M + Na]^+$: 696.2110, found: 696.2116.

4.2.8. *N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-[(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→2)-(1,4-triazole-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannosyl)-L-threonine benzyl ester 17

A solution of αManN_3-FmocThrOBn **5** (44 mg, 0.06 mmol, 1.0 equiv), 2'-propynyl-2-acetamide-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranoside **6** (23 mg, 0.06 mmol, 1.0 equiv), sodium ascorbate (2 mg, 0.0125 mmol, 0.5 equiv.) and $CuSO_4$ (1M, 6 µL, 0.006 mmol, 0.1 equiv) in DMF (0.5 mL) was inserted into a microwave tube and nextly submitted to microwave irradiation for 30 min. (100 °C, 150 W). The reaction was followed by TLC (EtOAc) and after completion the reaction mixture was

concentrated under reduced pressure and purified by silica gel column chromatography (gradient EtOAc/hexane 7:3, v/v → EtOAc) to afford the desired αGlcNAc-1-*O*-triazol-2Manα-ThrOBn **17** (48.1 mg, 0.042 mmol) in a 72% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.91 (s, 1H, H-triazol), 7.79 (d, 2H, *J* 7.4 Hz, C*H* Fmoc arom.), 7.64 (d, 2H, *J* 7.3 Hz, C*H* Fmoc arom.), 7.45-7.29 (m, 9 H, C*H* Fmoc arom, C*H* OBn arom), 6.12 (d, 1H, *J* 9.3 Hz, N*H*Ac), 5.71 (d, 1H, *J* 9.3 Hz, N*H* Thr), 5.46 (dd, 1H, $J_{1',2'}$ 5.1 Hz, $J_{2',3'}$ 9.9 Hz, H-2), 5.31-5.21 (m, 4H, H-3, H-4, H-3', H-4'), 5.19 (d, 1H, $J_{1,2}$ 5.1 Hz, H-1), 5.13 (d, 2H, *J* 12.8 Hz, CH_2OBn), 4.98 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1'), 4.90 (d, 1H, *J* 12.5 Hz, CH_{2a}-triazol), 4.66 (d, 1H, *J* 12.5 Hz, CH_{2b}-triazol), 4.55 (d, 1H, *J* 9.3 Hz, C*H*αThr), 4.46-4.23 (m, 6H, H-2', H-5, H-5', CH_2Fmoc, C*H*βThr), 4.12 (dt, 4H, $J_{5,6a/6b}$ 4.6 Hz, $J_{5',6a'/6b'}$ 7.2 Hz, H-6a, H-6b, H-6a', H-6b'), 2.12, 2.11, 2.07, 2.05, 2.03, 2.02, 2.00 (7 s, 21H, 7xCOCH_3), 1.35 (d, 3H, *J* 6.2 Hz, CH_3Thr). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2 (*C*O Fmoc), 170.7-169.3 (*C*OCH_3), 169.3 (*C*O OBn), 143.6-141.3 (*C*quat. Fmoc Ph), 134.9 (*C* quat.OCH_2Ph), 128.8-122.7 (*C*H Ph), 120.0 (CH-triazol), 98.4 (C-1), 96.8 (C-1'), 71.2 (C-3), 69.2 (C-3'), 68.8 (C-4), 68.3 (C-2), 68.2 (C-6), 67.8 (O*C*H_2 OBn), 67.4 (O*C*H_2 Fmoc), 65.2 (C-5'), 62.1 (*C*HβThr), 61.9 (C-6'), 60.9 (C-5), 60.6 (*O*CH_2-triazol), 60.4 (C-4'), 58.4 (*C*HαThr), 51.8 (C-2), 47.2 (*C*H Fmoc), 23.0 (*C*H_3CONH), 20.7-20.5 (CO*C*H_3), 17.7 (*C*H3Thr). ESI-HRMS: calcd. for $C_{55}H_{64}N_5O_{21}[M + H]^+$: 1030.4088, found: 1030.4066.

4.2.9. *N*-(Fluoren-9-ylmethoxycarbonyl)-*O*-[(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→2)-(1,4-triazole-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannosyl)-L-threonine 1

To a solution of compound **17** (14 mg, 0.012 mmol, 1.0 equiv) in MeOH (1.0 mL) was added glacial AcOH (0.1 mL) and 10% Pd/C (4.5 mg). The reaction mixture was stirred and kept under H_2 (1.5 atm) for approximately 30 min., being subsequently filtered through Celite, concentrated *in vacuo* and purified by column chromatography (DCM/MeOH 9:1, v/v). The product **1** was obtained in 82% yield (10.3 mg; 0.009 mmol). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.95 (broad s, 1H, H-triazol), 7.77 (d, 2H, *J* 7.3 Hz, C*H* Fmoc arom.), 7.64 (dd, 2H, *J* 6.6 Hz, *J* 9.3 Hz, C*H* Fmoc arom.), 7.41-7.33 (m, 4 H, C*H* Fmoc

arom.), 5.46 (dd, 1H, $J_{1,2}$ 2.4 Hz, $J_{2,3}$ 8.6 Hz, H-2), 5.80-5.72 (m, 1H, H-3), 5.55-5.49 (m, 1H, H-3'), 5.24-5.09 (m, 5H, H-1, H-1', H-4, H-4', CH_{2a}-triazol), 4.88 (dd, 1H, *J* 3.5 Hz, *J* 7.4 Hz, C*H*-Fmoc), 4.63 (d, 1H, *J* 12.3 Hz, CH_{2b}-triazol), 4.40-4.23 (m, 7H, H-2', H-5, H-5', CH_2Fmoc, C*H*αThr, C*H*βThr), 4.11 (d, 4H, $J_{6a/6b\,=\,6a'/6b'}$ 12.6 Hz, H-6a, H-6b, H-6a', H-6b'), 2.12-1.89 (m, 30H, 10xCOCH_3), 1.36 (d, 3H, *J* 5.8 Hz, CH_3Thr). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.2 (*C*O Fmoc), 170.7-169.3 (*C*OCH_3), 143.6-141.3 (*C*quat. Fmoc Ph), 134.9 (*C* quat.OCH_2Ph), 127.5-122.7 (*C*H Ph), 123.1 (CH-triazol), 98.4 (C-1), 96.8 (C-1'), 91.4 (C-2), 77.3 (C-3), 70.4 (C-3'), 69.4 (C-4), 69.1 (C-2'), 68.6 (C-6), 68.6 (O*C*H_2 Fmoc), 67.8 (C-5'), 65.3 (*C*HβThr), 61.9 (C-6'), 61.7 (C-5), 60.6 (*O*CH_2-triazol), 60.4 (C-4'), 60.1 (*C*HαThr), 47.7 (*C*H Fmoc), 22.4 (*C*H_3CONH), 20.7-20.5 (CO*C*H_3), 18.3 (*C*H_3Thr). ESI-HRMS: calcd. for $C_{48}H_{58}N_5O_{21}[M + H]^+$: 1040.3619, found: 1040.3441.

4.2.10. 3,6-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranoside-1-*O*-(methyl-1H-1,2,3-triazol-4-yl)-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester 18

A solution of αManN_3-FmocThrOBn **5** (42.4 mg, 0.057 mmol, 1.0 equiv.), 2'-propynyl-Gal-β1,4-αGlcNAc **7** (38.4 mg, 0.057 mmol, 1.0 equiv.), sodium ascorbate (5.6 mg, 0.028 mmol, 0.5 equiv.) and $CuSO_4$ (1M, 5.7 µL, 0.0057 mmol, 0.1 equiv) in DMF (0.5 mL) was inserted into a microwave tube and nextly submitted to microwave irradiation for 30 min (100 °C, 150 W). The reaction was followed by TLC (EtOAc) and after completion the reaction mixture was concentrated under reduced pressure and purified by silica gel column chromatography (EtOAc) to afford the desired Gal-β1,4-αGlcNAc-1-O-triazol-2Manα-ThrOBn **18** (28.3 mg, 0.019 mmol, 35%). ^{1}H NMR (500 MHz, $CDCl_3$): 7.88 (s, 1H, H-triazol), 7.77 (d, 2H, *J* 7.3 Hz, C*H* Fmoc arom.), 7.64 (d, 2H, *J* 7.3 Hz, C*H* Fmoc arom.), 7.43-7.27 (m, 9H, C*H* Fmoc arom, C*H* OBn arom), 5.70 (d, 1H, *J* 9.0 Hz, N*H*Ac), 5.66 (d, 1H, *J* 8.8 Hz, N*H* Thr), 5.44 (dd, 1H, $J_{3'',4''}$ 4.8 Hz, $J_{2'',3''}$ 9.8 Hz, H-3''), 5.34-5.30 (m, 3H, H-4'', H-3, H-4), 5.26 (d, 2H, *J* 11.9 Hz, CH_2OBn), 5.18-5.08 (m, 4H, H-2, H-3', H-4', H-4''), 5.04 (broad s, 1H, H-1), 4.95-4.94 (dd, 1H, $J_{1',2'}$ 3.4 Hz, $J_{2',3'}$ 10.5 Hz, H-2'), 4.89-4.84 (m, 2H, CH_{2a}-triazol, H-1'), 4.66 (d, 1H, *J* 12.8 Hz, CH_{2b}-triazol), 4.57

(d, 1H, $J_{1",2"}$ 8.0 Hz, H-1"), 4.51-4.37 (m, 4H, H-2", CHFmoc, C*H*αThr, C*H*β-Thr), 4.31-4.23 (m, 3H, H-5, CH_2Fmoc), 4.19-4.14 (m, 4H, H-6a', H-6b', H-6ª'', H-6b''), 4.12-4.08 (m, 1H, H-6b), 3.99 (dd, 1H, $J_{5,6a}$ 7.7 Hz, $J_{6a,6b}$ 11.1 Hz, H-6a), 3.86 (t, 1H, $J_{5'',6a''/6b''}$ 6.5 Hz, H-5''), 3.78 (t, 1H, $J_{5',6a'/6b'}$ 9.5 Hz, H-5'), 2.15-1.96 (m, 30H, 10xCOCH_3), 1.34 (d, 3H, *J* 6.2 Hz, CH_3Thr). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.2 (*C*O Fmoc), 170.7-169.3 (*C*OCH_3), 169.3 (*C*O OBn), 143.6-141.3 (*C*quat. Fmoc Ph), 134.9 (*C* quat. OCH_2Ph), 128.8-120.1 (*C*H Ph), 122.9 (CH-triazol), 101.0 (C-1"), 98.7 (C-1), 98.3 (C-1'), 92.6 (C-2), 77.8 (C-6"), 75.6 (C-6), 74.2 (C-5'), 70.9 (C-2), 69.9 (C-3), 70.6 (C-6'), 69.9 (C-4"), 69.0 (C-3'), 68.8 (C-5"), 68.6 (C-4), 68.3 (C-3"), 67.8 (O*C*H_2 OBn), 67.4 (O*C*H_2 Fmoc), 61.5 (*C*HβThr), 60.8 (C-5), 61.1 (*O*CH_2-triazol), 60.7 (C-4'), 61.1 (*C*HαThr), 51.3 (*C*H Fmoc), 49.9 (C-2"), 23.3 (*C*H_3CONH), 20.6-20.4 (CO*C*H_3), 17.7 (*C*H_3Thr). ESI-HRMS: calcd. for $C_{67}H_{80}N_5O_{29}$ $[M + H]^+$: 1418.4933, found: 1418.4845.

4.2.11. 3,6-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranoside-1-*O*-(methyl-1H-1,2,3-triazol-4-yl)-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine 2

To a solution of compound **18** (28 mg, 0.02 mmol, 1.0 equiv) in MeOH (3.0 mL) was added glacial AcOH (0.1 mL) and 10% Pd/C (15 mg, 0.06 mmol, 3.0 equiv). The reaction mixture was stirred and kept under H_2 (1.5 atm) for approximately 2 hours, being subsequently filtered through Celite, concentrated *in vacuo* and purified by column chromatography (DCM/MeOH 9:1, v/v). The product **2** was obtained in 97% yield (26.7 mg; 0.019 mmol). ^{1}H NMR (500 MHz, CD_3OD): δ 8.23 (s, 1H, H-triazol), 7.80 (d, 2H, *J* 7.4 Hz, C*H* Fmoc arom.), 7.71 (t, 2H, *J* 7.1 Hz, C*H* Fmoc arom.), 7.41-7.31 (m, 4 H, C*H* Fmoc arom.), 5.57 (dd, 1H, $J_{1,2}$ 4.9 Hz, $J_{2,3}$ 10.0 Hz, H-2), 5.37-5.34 (m, 2H, H-3, H-4"), 5.31 (t, 1H, $J_{2',3'} = J_{3',4'}$ 10.0 Hz, H-3'), 5.24-5.21 (m, 1H, H-4'), 5.12 (dd, 1H, $J_{3",4"}$ 3.5 Hz, $J_{2",3"}$ 10.2 Hz, H-3"), 5.05-5.01 (m, 2H, H-4', H-2"), 4.74-4.71 (m, 2H, H-1, H-2'), 4.68 (d, 1H, $J_{1",2"}$ 8.9 Hz, H-1"), 4.52-4.46 (m, 2H, C*H*αThr, C*H*βThr), 4.37-4.32 (m, 3H, H-5", CH_2Fmoc), 4.28-4.24 (m, 3H, H-6a", H-6b", C*H*Fmoc), 4.21 (d, 2H, *J* 12.5 Hz, CH_2-triazol), 4.13-4.07 (m, 4H, H-6a, H-6b, H-6a', H-6b'), 3.97 (d, 2H, $J_{5,6a} = J_{5',6a'}$ 6.6 Hz , H-5,

H-5'), 2.11-1.91 (m, 30H, 10xCOC*H*$_3$), 1.32 (d, 3H, *J* 6.5 Hz, C*H*$_3$Thr). ^{13}C NMR (125 MHz, CD_3OD): δ 171.4 (*C*O Fmoc), 171.1-169.9 (*C*OCH$_3$), 144.0-141.3 (*C*quat. Fmoc Ph), 127.5-119.6 (*C*H Ph), 124.2 (CH-triazol), 100.8 (C-1"), 98.6 (C-1), 98.2 (C-1'), 74.1 (C-5), 70.8 (C-3"), 70.1 (C-6), 70.0 (C-4'), 69.3 (C-4), 68.8 (C-5'), 68.7 (C-2), 67.2 (C-3), 66.7 (*C*HαThr), 65.0 (C-3'), 62.5 (O*C*H$_2$ Fmoc), 62.5 (C-6"), 62.3 (C-5''), 61.0 (C-6'), 60.7 (C-4"), 60.2 (C-2"), 59.8 (C-2'), 59.7 (*O*CH$_2$-triazol), 49.8 (*C*HβThr), 47.0 (*C*H Fmoc), 21.1 (*C*H$_3$CONH), 19.8-19.2 (CO*C*H$_3$), 17.8 (*C*H3Thr). ESI-HRMS: calcd. for $C_{60}H_{73}N_5NaO_{29}[M + Na]^+$: 1350.4283, found: 1350.4281.

4.2.12. General method for glycopeptide synthesis

The solid-phase synthesis of glycopeptides **3** and **4** were assembled manually using a fritted glass reaction vessel. FmocGly-Wang resin (100 mg) was swollen in CH_2Cl_2 for 2 h and then washed with DMF (3×). Deprotection of the *N*Fmoc group was performed using 50% morpholine-DMF followed by filtration and washing with DMF (3x), whilst coupling reactions were carried out with 2.0 mol equiv of Fmoc-amino acid and coupling reagents (PyBOP and HOBt) dissolved in DMF, and the base DIPEA. The coupling times varied from 24 h for the amino acids to 48 h and 68 h for the glycosyl-amino acids **1** and **2**. The reaction mixtures were filtered after each coupling and the resin was washed three times with DMF, CH_2Cl_2 and MeOH. After drying, small aliquots of resin (1 mg) were treated with 50% morpholine-DMF for removal of *N*Fmoc group, with consequent formation of dibenzofulvene product, allowing spectrophotometric detection of coupling reactions at 290 nm. After the last removal of *N*Fmoc group, *N*-acetylation reactions of glycopeptides were performed using Py and Ac_2O, followed by their cleavage from resin using an aqueous mixture of trifluoracetic acid and triisopropylsilane (TFA:TIPS:H_2O, 90:5:5). The obtained solutions were then filtered, concentrated and treated with 1M sodium methoxide solution in MeOH for removal of the sugar acetyl groups. The purification of the crude glycopeptides **3** and **4** was carried out on Shimadzu Shim-PaK HPLC equipment using a semi-preparative C18 reverse-phase column Shim-PacK CLC-ODS (M) column (250 x 10 mm), under gradient conditions (A: 0.1% TFA/ H_2O, B: MeCN, 10-70% B, within 40 min.), at a flow rate of 3.0 mL min.$^{-1}$, with UV

detection (224 nm). Upon these conditions the retention times for glycopeptides **3** and **4** were 15 min. and 11 min., respectively.

4.2.12.1. NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH 3

Using the SPPS method previously described, followed by purification by HPLC, glycopeptide **3** was obtained as an amorphous solid (3.2 mg, 2.8 µmol, 23 %). Rt (224 nm)= 14.3 min. ^{1}H NMR (500 MHz, D_2O): δ 8.10 (s, 1H, H-triazol), 4.99 (d, 1H, $J_{1,2}$ 5.6 Hz, H-1), 4.46 (d, 1H, $J_{1',2'}$ 4.1 Hz, H-1'), 4.28-4.26 (m, 2H, 2x CHαThr), 4.20-3.96 (m, 11H, CHαIle, CHαVal, CHβThr, CH_2Gly, H-4, H-4', H-3, H-3', H-2, H-2'), 3.90 (d, 1H, *J* 8.0 Hz, CHβThr), 3.79-3.76 (m, 3H, H-6a, H-6b, CH_{2a} triazol), 3.69-3.66 (m, 3H, H-6a', H-6b', CH_{2b} triazol), 3.59-3.51 (m, 1H, H-5'), 3.37 (t, 1H, $J_{5,6a}$ 9.0 Hz, H-5), 3.08 (t, 3H, *J* 6.5 Hz, CHαArg, CH_2δArg), 1.96-1.87 (m, 15H, 2x NHAc, CH_3Thr, 2x CH_3Val), 1.78-1.53 (m, 8H, CH_2βArg, CH_2γArg, CH_2γIle, CHβIle, CHβVal), 1.18-1.06 (m, 9H, CH_3Thr, 2x CH_3Ile). ESI-HRMS: calcd for $C_{46}H_{80}N_{13}O_{20}$ $[M + H]^+$: 1134.5637, found: 1134.5639.

4.2.12.2. NHAcThrVal[Gal-β1,4-αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH 4

Using the SPPS method previously described, followed by purification by HPLC, glycopeptide **4** was obtained as an amorphous solid (3.4 mg, 2.4 µmol, 12 %). Rt (224 nm)= 9.2 min. ^{1}H NMR (500 MHz, D_2O): δ 8.15 (s, 1H, H-triazol), 5.13 (s, 1H, H-4''), 5.0 (d, 1H, $J_{1,2}$ 4.7 Hz, H-1), 4.62 (d, 2H, *J* 12.3 Hz, CH_2 triazol), 4.47 (d, 1H, $J_{1',2'}$ 3.9 Hz, H-1'), 4.35 (d, 1H, $J_{1'',2''}$ 7.8 Hz, H-1"), 4.29 (dd, 1H, $J_{1,2}$ 5.7 Hz, $J_{2,3}$ 8.4 Hz, H-2), 4.25-4.22 (m, 2H, 2x CHαThr), 4.20-3.98 (m, 11H, CHαIle, CHαVal, CHβThr, CH_2Gly, H-4, H-4', H-3, H-3', H-2, H-2'), 3.88 (d, 1H, *J* 10.6 Hz, CHβThr), 3.83-3.76 (m, 5H, H-3'', H-6a, H-6b, H-6a',H-6b'), 3.68-3.61 (m, 4H, H-4", H-5", H-6a", H-6b"), 3.57-3.55 (m, 2H, H-5, H-5'), 3.43 (dd, 1H, $J_{1",2"}$ 8.0 Hz, $J_{2",3"}$ 9.6 Hz, H-2"), 3.12-3.08 (m, 3H, CHαArg, CH_2δArg), 1.97-1.92 (m, 9H, 2x NHAc, CH_3Thr), 1.75-1.24 (m, 14H, CH_2βArg, CH_2γArg, CH_2γIle, CHβIle, CHβVal, 2x CH_3Ile), 11.19-

1.07 (m, 9H, CH_3Thr, 2x CH_3Val). ESI-HRMS: calcd. for $C_{52}H_{90}N_{13}O_{25}^+$ $[M + H]^+$: 1296.6165, found: 1296.6166.

4.2.13. Enzymatic galactosylation of glycopeptide 3

The enzyme bovine β-1,4-galactosyltransferase (2.0 U/mL, EC 2.41.22, Sigma-Aldrich) was added to a solution of glycopeptide **3** (1.4 mg, 1.26 µmol) and UDP-galactose (0.38 mg, 0.63 µmol), in HEPES buffer (0.4 mL, 50 mM, pH 7.0, containing 100 mM $MnCl_2$). The mixture was incubated for 120 h at 37 °C, and was subsequently ultrafiltered against deionized water using a membrane of 3 kDa. The resulting product was dissolved in water and purified by gel filtration chromatography (TOYOPEARL HW-405, 85 cm x 15 mm) using 0.1% TFA in water, at a flow rate of 1.0 mL $min.^{-1}$

4.3. Molecular modeling studies

The initial structure of the natural glycopeptide Thr-Val-Thr[βGlcNAc-1,2αMan]-Ile-Arg-Gly from α-dystroglycan was obtained from the GlycamWeb server [26]. Then, this structure was modified to substitute βGlcNAc for αGlcNAc and to add the CH_2-triazole group between GlcNAc and Man with PyMOL to yield the glycopeptide analog **3**. The newly modified portion of the molecule was optimized at the semi-empirical level using the PM7 hamiltonian considering water as the implicit solvent with MOPAC [27] prior to docking.

The crystallographic structure of the bovine β-1,4-Galactosyltransferase 1 (β1,4GalT1) in which it is complexing the UDP-Galactose (UDP-Gal) and the Mn^{2+}cofactor was used (PDB ID 1TVY) [25]. The M344H mutation was not reversed since it is not expected to alter the acceptor site. Hydrogens were added with the open source version of PyMOL.

Docking calculations were performed with AutoDock Vina [28] with exhaustiveness parameter of 1000. The search volume was focused on the active site and the docking protocol was validated by reproducing the experimentally observed position of Chitobiose at the acceptor site as shown by the crystallographic structure PDB ID 1TW5 [25]. No water molecules were necessary to reproduce the

experimental data. Both glycopeptides were docked to the β1,4GalT1–Mn–UDPGal complex using the validated protocol.

Supporting Information Available: The Supporting Information comprises the ^{1}H NMR, ^{13}C NMR, HSQC and ESI-MS analyses of the main compounds, as well as a complementary figure related to molecular modeling studies.

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Highlights

- α-Dystroglycan mucins analogs may act as substrates for glycosyltransferases and as tools for antibodies generation.
- Novel triazole-derived glycopeptides were synthesized as analogs of α-dystroglycan mucins.
- The triazole-glycopeptide NHAcThrVal[αGlcNAc-1-triazol-2Manα]ThrIleArgGlyOH acted as inhibitor of the enzyme bovine β-1,4-galactosyltransferase.