Chemoenzymatic Synthesis of Biotinylated Nucleotide Sugars as Substrates for Glycosyltransferases

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*The enzymatic oxidation of uridine 5′-diphospho-α-D-galactose (UDP-Gal) and uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine (UDP-GalNAc) with galactose oxidase was combined with a chemical biotinylation step involving biotin-ε-amidocaproylhydrazide in a one-pot synthesis. The novel nucleotide sugar derivatives uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-galactose (UDP-6-biotinyl-Gal) and uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-*N*-acetyl-α-D-galactosamine (UDP-6-biotinyl-GalNAc) were synthesized on a 100-mg scale and characterized by mass spectrometry (fast atom bombardment and matrix-assisted laser desorption/ionization time of flight) and one/two dimensional NMR spectroscopy. It could be demonstrated for the first time, by use of UDP-6-biotinyl-Gal as a donor substrate, that the human recombinant galactosyltransferases β3Gal-T5, β4Gal-T1, and β4Gal-T4 mediate biotinylation of the neoglycoconjugate bovine serum albumin – p-aminophenyl* N*-acetyl-β-D-glucosaminide (BSA – $(GlcNAc)_{17}$) and ovalbumin. The detection of the biotin tag transferred by β3Gal-T5 onto BSA – $(GlcNAc)_{17}$ with streptavidin – enzyme conjugates gave detection limits of 150 pmol of tagged GlcNAc in a Western blot analysis and 1 pmol of tagged GlcNAc in a microtiter plate assay. The degree of Gal-biotin tag transfer onto agalactosylated hybrid N-glycans present at the single glycosylation site of ovalbumin was dependent on the Gal-T used (either β3Gal-T5, β4Gal-T4, or β4Gal-T1), which indicates that the acceptor specificity may direct the transfer of the Gal-biotin tag. The potential of this biotinylated UDP-Gal as a novel donor substrate for human galactosyltransferases lies in the targeting of distinct acceptor structures, for example, under-galactosylated glycoconjugates, which are related to diseases, or in the quality control of glycosylation of recombinant and native glycoproteins.*

KEYWORDS:

carbohydrates · enzyme catalysis · glycoproteins · glycosyltransferases · nucleotide sugars

Introduction

D-Galactose (Gal) and *N*-acetyl-D-galactosamine (GalNAc) are ubiquitous constituents of oligosaccharide chains of soluble and cell surface bound glycoconjugates with important biological functions.[1] In previous studies these components were targeted by various labeling techniques for oligosaccharide chains in glycoconjugates.[2] The labeling of glycoconjugates by radioactive tracers, fluorescent dyes, or biotin derivatives through the Gal or the GalNAc moieties was accomplished by the oxidation of the primary alcohol groups of the monosaccharides with galactose oxidase (EC 1.1.3.9) and subsequent conversion of the aldehydes with, for example, tritiated sodium borohydride ($NaBT_4$) or hydrazide reagents carrying the label.[3, 4] However, these methods resulted in a random and rather unselective distribution of the label within the oligosaccharide chains of a glycoprotein or glycolipid.

In view of the important role of glycoconjugates in biological recognition (for example, cell – cell adhesion), acute and chronic diseases (such as inflammation) and numerous human cancer types, techniques capable of introducing a label at a unique site in the oligosaccharide chain of the relevant glycoproteins and glycolipids are highly desirable. These selective methods would facilitate the tracing of aberrant glycosylation patterns associated with diseases, as well as the analysis or diagnosis of specific glycoconjugates related to biological recognition, and pharmaceutical therapy.

The selectivity of one labeling method has already been demonstrated by the transfer of nucleotide sugar derivatives with glycosyltransferases. Fluorescent-labeled or tagged cytosine 5′-monophospho-neuraminic acid (CMP-Neu5Ac) and guanosine 5′-diphospho-β-L-fucose (GDP-Fuc) were transferred by

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sialyltransferases[5–7] or by a fucosyltransferase from milk,[8] respectively. Labeling of cell surfaces was also realized by the transfer of GDP-Fuc derivatives substituted in the C-6 position with a blood group trisaccharide or selectin ligand oligosaccharides.[9, 10] Metabolic cell-surface engineering was achieved by adding derivatives of *N*-acetylmannosamine, for example, *N*-levulinoylmannosamine and *N*-azidoacetylmannosamine, into the biosynthetic pathway of Neu5Ac. Chemoselective coupling steps target the azide- or carbonyl-functionalized Neu5Ac transferred in vivo by cell-specific sialyltransferases.[11–14] Recently, it was demonstrated that feeding of Chinese hamster ovarian (CHO) cells with 2-deoxy-2-acetonyl-D-galactose resulted in the exposure of this 2-keto isosteric derivative of GalNAc on the cell surfaces.[15]

Similar derivatives of uridine 5′-diphospho-α-D-galactose (UDP-Gal; **1**) and uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine (UDP-GalNAc; **2**) for transfer reactions with galactosyl- or *N*-acetylgalactosaminyltransferases (Gal-Ts or GalNAc-Ts) have not yet been developed. Great potential for the selective transfer of a labeled nucleotide sugar onto a specific acceptor structure in a glycoprotein or glycolipid lies in the enzymes of the recently identified glycosyltransferase families: the β3-galactosyltransferases (β3-Gal-Ts),[16–20] the β4-galactosyltransferases (β4-Gal-Ts),[16, 21–28] and the polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts).[29–37] Specific characteristics of these glycosyltransferases could contribute to a selective targeting of glycoconjugates by a glycosyltransferase-mediated labeling technique; these characteristics are their specificity for certain acceptor structures in glycoproteins and glycolipids, their tissue- and cell-type specific expression, and their relationship to the exposure of carbohydrate ligands, tumor-associated antigens, and aberrant glycosylation patterns.[38–42] However, these enzymes have yet not been tested for their acceptance of labeled or tagged UDP-Gal(NAc).

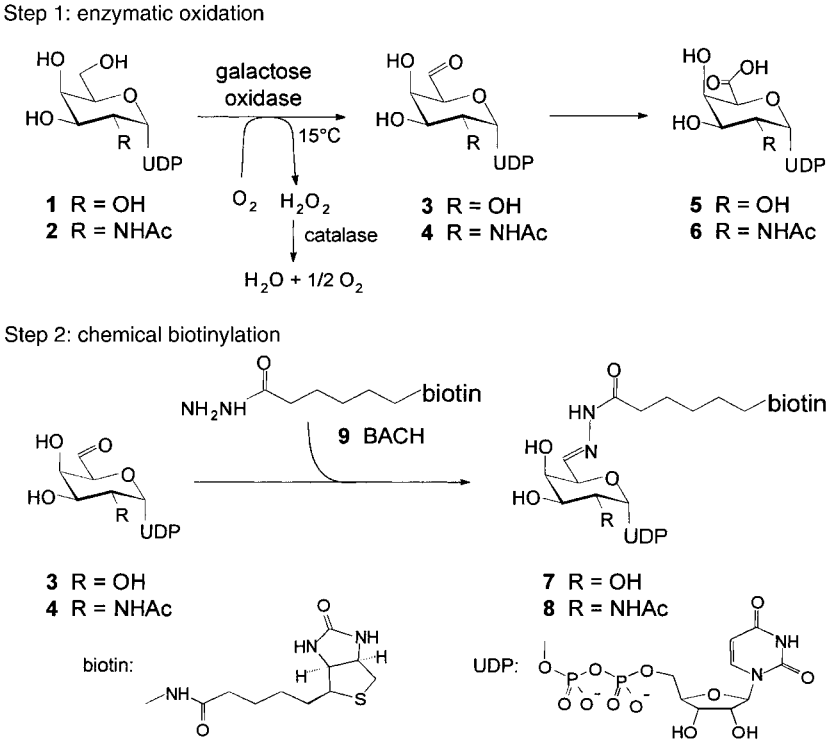
In this context the aim of our long-term studies is to develop tagged or labeled derivatives of UDP-Gal and UDP-GalNAc. Radioactive-labeled UDP-Gal and UDP-GalNAc derivatives have already been synthesized by the oxidation of the primary alcohol group with galactose oxidase and subsequent reduction of the aldehydes by $NaBT_4$.[43–45] The synthesis of nonradioactive-labeled (for example, fluorescent-labeled) or tagged (for example, with biotin) UDP-Gal and UDP-GalNAc would be very attractive for diagnostic, synthetic, and analytical applications. However, the donor specificity of most of the glycosyltransferase family members has not been investigated so far except for that of β4Gal-T1, which was previously characterized to accept 6-deoxy- and 6-fluoro-Gal.[46]

The present paper describes the preparative one-pot chemoenzymatic synthesis of biotinylated UDP-Gal and UDP-GalNAc compounds, on a 100-mg scale. The analytical labeling method for Gal and GalNAc employing galactose oxidase and a biotin hydrazide[4] was adapted to the preparative synthesis of nucleotide sugar derivatives. The products were tested as donor substrates for different recombinant human Gal-Ts and ppGalNAc-Ts. The first proof of the concept of a galactosyltransferase-mediated tagging of glycoproteins was obtained by studies with the neoglycoprotein bovine serum albumin – p-aminophenyl *N*-acetyl-β-D-glucosaminide (BSA – $(GlcNAc)_{17}$) and ovalbumin, which were analyzed by Western blot analysis and an enzyme-linked streptavidin assay (ELSA).

Results and Discussion

Optimized conditions for the stepwise synthesis of UDP-6-biotin-hydrazono-Gal (7)

Scheme 1 outlines the strategy for the stepwise synthesis of the biotinylated UDP-Gal(NAc) targets. In order to optimize the reaction conditions concerning the enzymatic oxidation and chemical coupling to biotin-ε-amidocaproylhydrazide (BACH, **9**), UDP-Gal (**1**) was chosen as the substrate; it was presupposed that the results could be transferred to UDP-GalNAc (**2**). Preliminary investigations into the substrate spectrum of galactose oxidase indicated a residual activity of 32% and 9%,



Scheme 1. *Strategy for the stepwise chemoenzymatic synthesis of UDP-6-biotin-hydrazono-Gal(NAc) targets **7** and **8**. Step 1: Enzymatic oxidation of UDP-Gal (**1**) and UDP-GalNAc (**2**) by galactose oxidase. The aldehydes **3** and **4** are further converted into the corresponding uronic acids **5** and **6**, respectively. The second oxidation reaction inevitably limits the yield of the aldehydes **3** and **4** (Figure 1). Step 2: Chemical biotinylation of **3** and **4**, respectively, with biotin-ε-amidocaproylhydrazide (BACH, **9**) to form the corresponding UDP-6-biotin-hydrazono-Gal(NAc) compounds **7** and **8**. The yield for **7** was 85%, which results in an overall yield of 59%.*

respectively, for a concentration of 1.5 mM of **1** and **2**, with reference to D-galactose (data not shown). In contrast to other reported methods,[43–45] a pH value of 6.0 was used in order to stabilize the nucleotide sugars and to meet the optimum pH range of 6–7 for galactose oxidase.[47] A temperature of 15 °C was chosen as a good compromise between oxygen saturation and enzyme activity. Inactivation of galactose oxidase by the side product H_2O_2 was avoided by the presence of catalase degrading H_2O_2 to water and oxygen. However, under optimized reaction conditions the yield of the enzymatic oxidation product uridine 5′-diphospho-6-aldo-α-D-galactose (UDP-6-aldo-Gal, **3**) was limited to 75% after 90 min incubation (Figure 1). A side product **5** was simultaneously formed with a yield of 13% after 90 min and a maximum yield of 19% after 240 min (Figure 1). The identification of the side product **5** afforded a separate synthesis; after isolation it was identified by mass spectrometry and NMR spectroscopy as uridine 5′-diphospho-α-D-galacturonic acid (UDP-GalA, **5**). The oxidation of galactosides to galactosiduronic acids was described previously for methyl D-galactoside as a substrate of galactose oxidase,[48] and it was observed then that the formation of the uronic acid started after the formation of the sugar aldehyde was completed. Therefore, the accumulation of the side product could be avoided by terminating the reaction in time. However, the present results for the conversion of **1** showed the simultaneous formation of UDP-6-aldo-Gal (**3**) and UDP-GalA (**5**) during the synthesis, in a manner which was not controllable by variation in the incubation time (Figure 1).

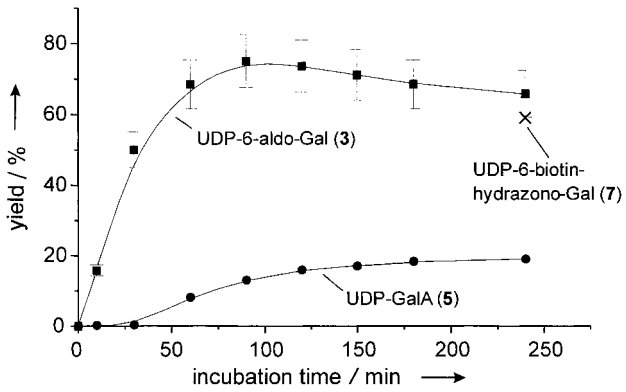
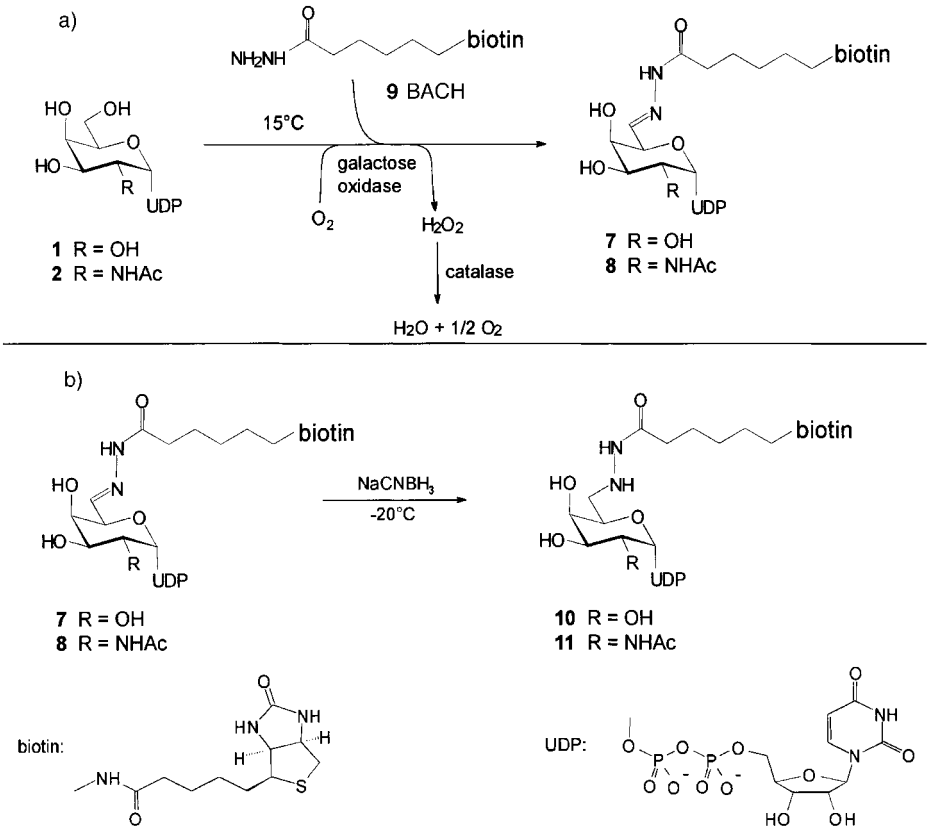


Figure 1. *Time-course graph of the oxidation of UDP-Gal **1** with galactose oxidase. A maximum yield of 75% was reached for UDP-6-aldo-Gal (**3**) after 90 min. The side product UDP-GalA (**5**) was simultaneously formed with a yield of 13% after 90 min and a maximum yield of 19% after 240 min. For further details, see the text and the Experimental Section.*

In a second chemical step **3** was selectively treated with **9** for 2 h after the proteins were removed from the reaction mixture by ultrafiltration (Scheme 1). Uridine 5′-diphospho-6-biotin-ε-amidocaproyl-hydrazono-α-D-galactose (UDP-6-biotin-hydrazono-Gal, **7**) was formed in 85% yield. The overall yield for the stepwise synthesis of **7** was 59% (Scheme 1).

One-pot chemoenzymatic synthesis of UDP-6-biotin-hydrazono-Gal (7) and reduction to UDP-6-biotinyl-Gal (10)

The combination of the enzymatic oxidation step with the chemical biotinylation step in a chemoenzymatic one-pot reaction avoided the formation of the side product UDP-GalA (**5**) completely. The overall yield of **7** was significantly increased to 78% by enzymatic oxidation of **1** followed by in situ conversion of the intermediate nucleotide sugar aldehyde **3** with **9** (Scheme 2a). To the best of our knowledge this is the first example for the synthesis of a nucleotide sugar derivative through such an approach. It is obvious from the high product yield that the hydrazide **9** does not inhibit the enzyme activity.



Scheme 2. *Chemoenzymatic synthesis of UDP-6-biotinyl-Gal(NAc) compounds **10** and **11**. A) One-pot chemoenzymatic synthesis of UDP-6-biotin-hydrazono-Gal(NAc) compounds **7** and **8**. The yields for **7** and **8** were 82% and 77%, respectively. The formation of uronic acids was not observed. B) Chemical reduction of **7** and **8** with $NaCNBH_3$ in frozen aqueous solution at −20 °C. The reaction yielded **10** and **11** in quantitative yields after 48 h. After product isolation the overall yields for **10** and **11** were 38% and 40%, respectively. For further details, see the Experimental Section.*

Compound **7** was subsequently stabilized by chemical reduction to the corresponding uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-galactose (UDP-6-biotinyl-Gal, **10**; Scheme 2b). The reaction conditions had to be optimized for this step. $NaBH_4$[49] and KBH_4,[50] which were previously used in similar labeling experiments under alkaline conditions, could not be applied because of the instability of the nucleotide sugars. Therefore, $NaCNBH_3$ was chosen for chemical reduction in a slightly acidic aqueous solution, in which the nucleotide sugars are more stable. The reaction was investigated at temperatures between −80 and 37 °C (Figure 2). A quantitative yield of **10** was

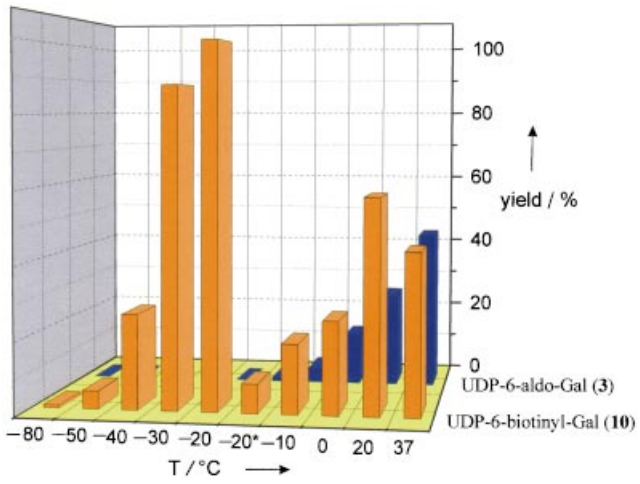


Figure 2. *Influence of the reaction temperature on the chemical reduction of UDP-6-biotin-hydrazono-Gal (**7**). The yields refer to the sum of the relative areas (100%) for **7**, **10**, and **3** in HPLC analysis. For further details, see the text and the Experimental Section.*

obtained in the frozen solid state at −20 °C. It should be noted that when freezing of the reaction solution at −20 °C was avoided by the addition of glycerol, the yield of **10** was only 8%. At all other temperatures UDP-6-biotin-hydrazono-Gal (**7**) was partially decomposed to yield **3** as well as the desired product **10** in variable yields, as analyzed by high-pressure liquid chromatography (HPLC; Figure 2). In conclusion, in the preparative syntheses a temperature of −20 °C and an incubation time of 48 h were chosen for the chemical reduction step (Scheme 2b).

Preparative synthesis of UDP-6-biotinyl-Gal (10) and UDP-6-biotinyl-GalNAc (11)

The one-pot chemoenzymatic syntheses of the UDP-6-biotinyl-Gal(NAc) targets, **10** and **11**, through the UDP-6-biotin-hydrazono-Gal(NAc) compounds, **7** and **8**, was scaled up to produce donor substrates for enzymatic transfer experiments. The main problem for synthesis on a 100-mg scale turned out to be the oxygen supply for the enzymatic oxidation reaction. The activity and the stability of the enzyme depend on the concentration of oxygen.[51, 52] To maintain an optimum activity and stability of the biocatalyst it was necessary to saturate the reaction solution with oxygen. However, a simple diffusive gas transport through the surface of the solution was no longer sufficient on the preparative scale. The yield and the reaction rate of the synthesis were low unless large amounts of enzyme were added. Alternatively, oxygen was blown into the reaction solution resulting in a very low enzyme stability ($t_{1/2} = 2.5$ h). The shear force at the gas–liquid interface produced by the gas bubbles inactivated the enzyme. Finally, it was found that the best methodology for gas supply was a bubble-free diffusive aeration with thin-walled hydrophobic silicon tubes, an approach recently introduced in enzymatic syntheses with cyclohexanone monooxygenase.[53] The combined chemoenzymatic synthesis in a reactor with integrated bubble-free aeration, whereby the solution of the stirred vessel was diverted through a silicon tube placed in a flask filled with oxygen, yielded 82% of **7** after 56 h. Subsequent quantitative reduction with $NaCNBH_3$ in frozen aqueous solution at −20 °C (Scheme 2b) and product isolation gave 128 mg (38%) of UDP-6-biotinyl-Gal (**10**). The yield in the synthesis of **8** was 77% after 65 h. Quantitative reduction and product isolation yielded 98 mg (40%) of UDP-6-biotinyl-GalNAc (**11**).

Stability of UDP-6-biotinyl-Gal (10) and UDP-6-biotinyl-GalNAc (11) in the presence of divalent metal ions

Since the activity of many Leloir glycosyltransferases is dependent upon divalent metal ions (mainly Mn^{2+}) as cofactors, the stability of **10** and **11** was tested in the presence of different divalent metal ions (data not shown). Both compounds were very sensitive to a low concentration of Mn^{2+}. With 0.1 mM Mn^{2+} ions in 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/NaOH buffer (pH 7.4), 63% of both **10** and **11** were decomposed after 10 h at 37 °C. In each case HPLC analysis revealed, as decomposition products, uridine 5′-monophosphate and another compound which elutes at a similar retention time to the corresponding nucleotide sugars **1** and **2**, respectively. However, **10** and **11** were more stable when the Mn^{2+} ions were replaced by Ni^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, and Ca^{2+} ions, respectively (data not shown). At concentrations of 0.1 mM of these divalent cations in 100 mM HEPES/NaOH buffer (pH 7.4), only 5–10% of **10** and **11** were decomposed as described above after 20 h at 37 °C.

UDP-6-biotinyl-Gal (10) as a donor substrate for the galactosyltransferases

Since the presence of Mn^{2+} ions should be avoided with **10**, the residual activities of bovine β4Gal-T1, human β4Gal-T2, -T3, and -T4, and human β3Gal-T5 were tested in the absence of Mn^{2+} ions. The relative activities with UDP-Gal turned out to be 20% for β4Gal-T1, -T2, -T3, and -T4, and 135% for β3Gal-T5 with reference to a standard assay with 5 mM Mn^{2+} ions (data not shown). Transfer studies were then performed in the absence of Mn^{2+} ions with **10** as the donor and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (GlcNAc-MU) as the acceptor substrate. Thin layer chromatography (TLC) analysis revealed the generation of a transfer product synthesized by β3Gal-T5 after 16 h of incubation. However, the results for the β4Gal-Ts were not clear by TLC analysis, and therefore HPLC analysis with UV

and fluorescence detection was applied. All chromatograms of the reactions with **10** as the donor and β4Gal-T2, -T3, or -T4 as the biocatalyst showed a new fluorescent peak that increased over 13 h of reaction time. However, product formation could not be detected with **10** as the donor substrate for bovine β4-Gal-T1. In summary, these results demonstrate that β4Gal-T2, β4Gal-T3, and β4Gal-T4, as well as β3Gal-T5 but not bovine β4-Gal-T1, accept **10** as a donor substrate for transfer onto GlcNAc-MU in the absence of Mn^{2+} ions.

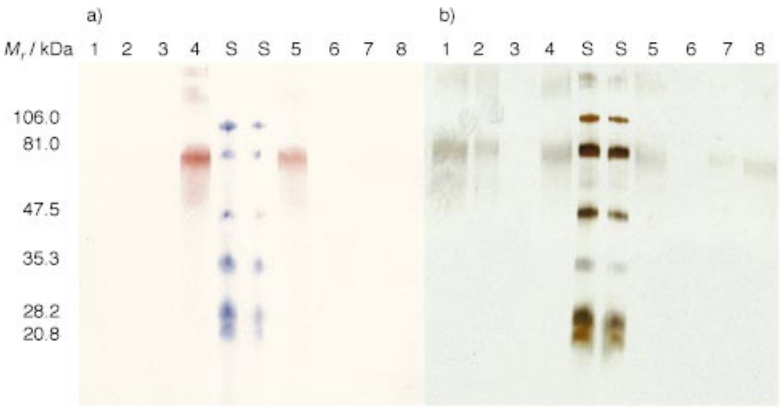


Figure 3. *Glycosyltransferase-mediated biotinylation of BSA – $(GlcNAc)_{17}$ by human β3Gal-T5, as analyzed by: a) Western blot analysis and b) SDS-PAGE. Lanes 1 and 8: control without enzyme; lanes 2 and 7: control with* ***1*** *as the donor substrate; lanes 3 and 6: control without an acceptor substrate; lanes 4 and 5: samples with* ***10*** *as the donor substrate. Samples in lanes 1 – 4 and 5 – 8 contain 1.6 μg and 1 μg of BSA – $(GlcNAc)_{17}$, respectively. The band detected in lane 5 of (a) corresponds to 152 pmol of biotin-labeled GlcNAc. Samples in the lanes labeled S contain a Coomassie-prestained protein standard (phosphorylase B (101), BSA (79), ovalbumin (50.1), carbonic anhydrase (34.7), soybean trypsin inhibitor (28.4), and lysozyme (20.8 kDa)). For further details, see the Experimental Section.*

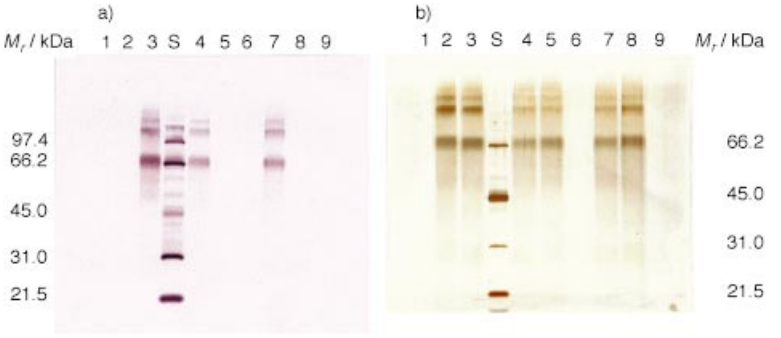


Figure 4. *Glycosyltransferase-mediated biotinylation of BSA – $(GlcNAc)_{17}$ by human β3Gal-T5, human β4Gal-T1, and human β4Gal-T4, as analyzed by: a) Western blot analysis and b) SDS-PAGE. Enzymes: Lanes 1 – 3: human β3Gal-T5; lanes 4 – 6: human β4Gal-T1; lanes 7 – 9: human β4Gal-T4. Samples in lanes 2 – 5, 7, and 8 contain 1.6 μg of BSA – $(GlcNAc)_{17}$. Lanes 1, 6, and 9: control without an acceptor substrate; lanes 2, 5, and 8: control with* ***1*** *as the donor substrate; lanes 3, 4, and 7: samples with* ***10*** *as the donor substrate. Samples in the lanes labeled S contain a biotinylated protein standard (phosphorylase B (97.4), BSA (66.2), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), lysozyme (14.4 kDa)). For further details, see the Experimental Section.*

Galactosyltransferase-mediated biotinylation of BSA – $(GlcNAc)_{17}$

The neoglycoconjugate BSA – $(GlcNAc)_{17}$, which carrys on average 17 μmol of β-D-GlcNAc per μmol of BSA, was chosen as a model protein. In an experiment with β3Gal-T5, HPLC analysis revealed a conversion of 25% (1.25 mM) of **10**, which corresponds to a conversion of 62% with reference to BSA-bound GlcNAc. A control experiment with **1** as the donor substrate gave a 100% conversion of the nucleotide sugar under identical reaction conditions. The samples were then analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with subsequent detection of 6-biotinyl-D-Gal(β1-3)GlcNAc(β1-*p*-aminophenyl-BSA) by a streptavidin – alkaline phosphatase conjugate (Figure 3a and b). Lane 5 in Figure 3a and b contains 1 μg (14.3 pmol) of BSA – $(GlcNAc)_{17}$ which corresponds to 243 pmol of GlcNAc residues. With 62% conversion of **10**, as analyzed by HPLC, the amount of biotin-labeled GlcNAc in lane 5 is 152 pmol. The control experiments with **1** as the donor substrate (lanes 2 and 7 in Figure 3a and b) lack the biotin label and gave no reaction with the streptavidin – alkaline phosphatase conjugate.

The transfer of the Gal-biotin tag from **10** onto BSA – $(GlcNAc)_{17}$ was also demonstrated with human β4Gal-T1, in contrast to the bovine enzyme, and for human β4Gal-T4. Figure 4a shows the human β4Gal-T1 and β4Gal-T4 mediated biotinylation of BSA – $(GlcNAc)_{17}$ (lanes 4 and 7) in comparison to β3Gal-T5. The main biotinylated protein band with an apparent molecular mass of 77 kDa corresponds to BSA – $(GlcNAc)_{17}$ with the Gal-biotin tag. In addition protein bands with higher apparent molecular masses appear, which are most likely due to artifacts of highly glycosylated BSA molecules poorly loaded with SDS. In the silver-stained gel the samples and the control experiments with donor **1** show the typical diffuse bands of the galactosylated neoglycoprotein (lanes 2 – 5, 7, 8 in Figure 4b).

Samples of BSA – $(GlcNAc)_{17}$ with the Gal-biotin tag were also analyzed by an ELSA in microtiter plates. Tagged BSA – $(GlcNAc)_{17}$ and appropriate controls were diluted and adsorbed onto microtiter plate wells. The adsorbed biotin-Gal(β1-3/4)GlcNAc-(β1-*p*-aminophenyl-BSA) was detected by binding of a streptavidin – horseradish peroxidase conjugate and subsequent reaction with an *o*-phenyldiamine (OPD) substrate. The dilution of the tagged BSA – $(GlcNAc)_{17}$ samples reveals that the transfer of **10** is slightly better with human β4Gal-T1 than with human β4Gal-T4 (Figure 5).

Figure 6a shows a hyperbolic saturation curve for the detection of BSA – $(GlcNAc)_{17}$ with the Gal-biotin tag formed by β3Gal-T5. The concentration of tagged GlcNAc residues was calculated as described above on the basis of the conversion of **10** as determined by HPLC. Within the given conditions, the optical density (OD) at 490 nm is linear for concentrations

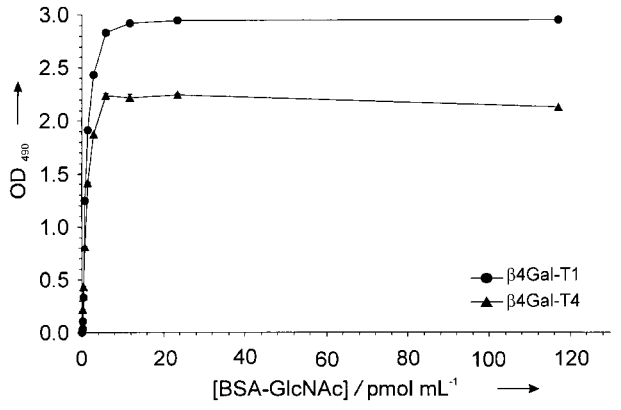


Figure 5. *Glycosyltransferase-mediated biotinylation of BSA – $(GlcNAc)_{17}$ by β4Gal-T1 and β4Gal-T4, as analyzed by an enzyme-linked streptavidin assay. The concentration of available GlcNAc residues (GlcNAc-R) was calculated in pmol. OD_{490} = optical density measured at 490 nm. For further details, see the text and the Experimental Section.*

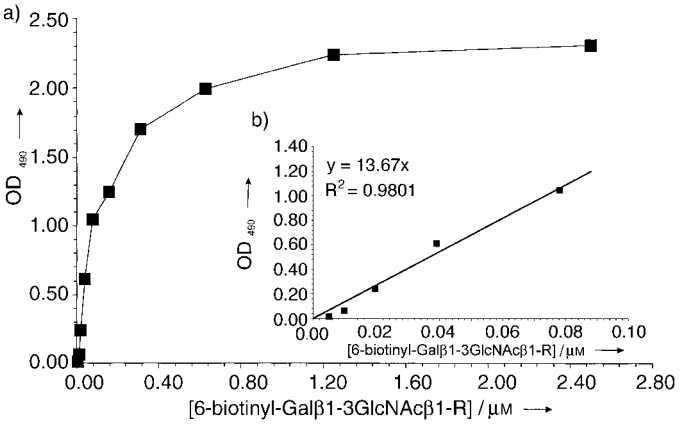


Figure 6. *Glycosyltransferase-mediated biotinylation of BSA – $(GlcNAc)_{17}$ by β3Gal-T5, as analyzed by an enzyme-linked streptavidin assay. a) The concentration of GlcNAc residues with the Gal-biotin tag were calculated according to the conversion of **10**, as analyzed by HPLC. b) The linear relationship between the concentration of GlcNAc residues with the Gal-biotin tag and the signal of the horseradish peroxidase reaction at OD_{490}. Within the given conditions, the assay results are linear for concentrations of biotin-Gal(β1-3)GlcNAc(β1-R) from 10 – 80 nmol L^{-1}, which correspond to 1 – 8 pmol in the microtiter wells. For further details, see the text and the Experimental Section.*

between 10 and 80 nmol L^{-1} of GlcNAc residues with the Gal-biotin tag, which corresponds to 1 – 8 pmol in the microtiter plate wells (Figure 6 b).

In conclusion, the experiments clearly prove that members of the β3-GalT and β4-GalT families accept UDP-6-biotinyl-Gal (**10**) as a novel donor by transfering the tagged galactose onto a neoglycoprotein.

Galactosyltransferase-mediated biotinylation of ovalbumin

The targeting of "under-galactosylated" N- and/or O-linked oligosaccharides of native or recombinant glycoproteins is one possible application of galactosyltransferase-mediated biotinylation. Ovalbumin was chosen as an example for a glycoprotein with only one N-linked oligosaccharide chain per molecule. Previous studies have shown that oligomannose and hybrid-type *N*-glycans occur at Asn293.[54, 55] The hybrid-type structures were found to expose terminal GlcNAc residues, and were thus largely "under-galactosylated".[55–57]

Ovalbumin was tested as an acceptor for galactosyltransferase-mediated biotinylation with β3Gal-T5, β4Gal-T1, and β4Gal-T4. The samples of ovalbumin with the Gal-biotin tag were diluted after the transfer experiment and analyzed by an ELSA. Figure 7 shows different levels of biotinylation for ovalbumin with the three enzymes. Biotinylation mediated by β3Gal-T5 gives the highest OD_{490} signal in the ELSA followed by β4Gal-T4. Under the conditions applied for the ELSA, the lowest detected concentration of ovalbumin was 11.7 nmol mL^{-1}. Figure 7 also shows that biotinylation with β4Gal-T1 was very low and close to the detection limit.

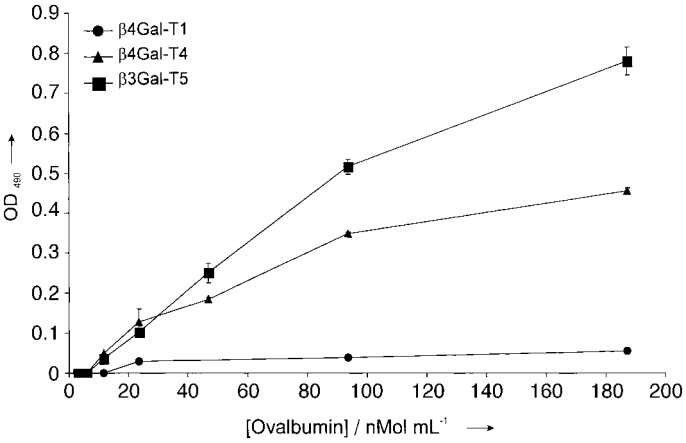


Figure 7. *Glycosyltransferase-mediated biotinylation of ovalbumin by human β3Gal-T5, human β4Gal-T1, and human β4Gal-T4, as analyzed by an enzyme-linked streptavidin assay. For further details, see the text and the Experimental Section.*

Unlike the results obtained with BSA – $(GlcNAc)_{17}$, the different results for biotinylation of ovalbumin by β3Gal-T5, β4Gal-T1, and β4Gal-T4 may be due to the distinct acceptor specificity of these members of the β3Gal-T and β4Gal-T families. However, *N*-glycans, such as those present on ovalbumin, were reported in literature to be very poor in vitro acceptor substrates of β3Gal-T5 and β4Gal-T4.[16] β3Gal-T5 prefers core 3 type *O*-glycans[58] and the glycosphingolipids Lc3Cer (GlcNAc(β1-3)Gal(β1-4)Glc(β1-ceramide) and Gb4 (GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-ceramide).[59] β4Gal-T4 transfers Gal onto core 2 type *O*-glycans[60] and onto the glycospingholipids Lc3Cer and nLc5Cer (GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-ceramide).[24] In contrast, it was recently reported that β4Gal-T4 is also involved in the *N*-glycan biosynthesis in vivo.[61] In this context UDP-6-biotinyl-Gal (**10**) may be an ideal donor substrate, which facilitates the reexamination of the individual acceptor specificity of the members of the Gal-T families by isolation and characterization of their galactosylated glycan products.

On the other hand, the relative poor acceptance of ovalbumin by β4Gal-T1 with **10** is surprising, since the hybrid-type *N*-glycans of ovalbumin were described as acceptor substrates with UDP-Gal (**1**).[24] The hybrid-type *N*-glycans seem to affect the transfer of the modified donor substrate **10** by human β4Gal-T1, whereas BSA – $(GlcNAc)_{17}$ has no effect. In this respect, the *p*-aminophenyl spacer of BSA – $(GlcNAc)_{17}$ may support acceptance by the enzyme. Further experiments with other acceptor structures of human β4Gal-T1, such as poly-*N*-acetyllactosamine chains of complex *N*-glycans,[60] should give more insight into the application of this enzyme.

In conclusion, further work on the kinetics of **10** and different acceptors will reveal the conditions for β3/β4Gal-T-mediated biotinylation of the *N*- and *O*-glycans of glycoproteins and glycolipids. Our preliminary studies clearly demonstrate that members of Gal-T families are able to mediate biotinylation of hybrid-type *N*-glycans on a glycoprotein with **10** as the donor substrate. The presented method may be further exploited for the detection of agalactosyl glycans of glycoproteins related to diseases such as rheumatoid arthritis,[42] immunoglobulin A (IgA) nephropathy,[62] and breast cancer. A diagnostic marker for patients with rheumatoid arthritis are agalactosyl complex type *N*-glycans of immunoglobulin G (IgG).[42, 63] Patients with IgA nephropathy show circulating IgA1 with agalactosyl *O*-glycans of the Tn-antigen type.[64] Deficient core 1 β3Gal-T activity in the *O*-glycosylation pathway of the mucin 1 glycoprotein leads to the exposure of the Tn-antigen, GalNAc(α1-*O*)Ser/Thr, as a diagnostic tumor marker for patients with breast cancer.[40, 65] In combination with fluorescent or chemiluminescent detection kits also, cell surfaces of benign or malignant cells may be targeted by UDP-6-biotinyl-Gal (**10**).

UDP-6-biotinyl-GalNAc (11) as the donor substrate of ppGalNAc-Ts

As shown before, **11** is decomposed in the presence of Mn^{2+} ions, but is sufficiently stable with other divalent cations. In order to adapt these conditions to transfer experiments with ppGalNAc-T1, -T2, and -T6, the enzymes were assayed with the donor substrate **2** in the absence and in the presence of divalent cations. The residual enzyme activity of ppGalNAc-T1, -T2, and -T6 without Mn^{2+} ions was only 4 – 10% of the activity with 20 mM Mn^{2+} ions (data not shown). The effect of cations other than Mn^{2+} on the activity of ppGalNAc-Ts was previously reported. Mg^{2+} and Ca^{2+} ions could not substitute for Mn^{2+} ions [30] whereas a porcine ppGalNAc-T was partially reactivated by the addition of 20 mM Co^{2+} or Ni^{2+} ions.[66] In the present study, the addition of Ni^{2+} ions had no effect on the activity of human ppGalNAc-T1, -T2, and -T6. In contrast, all three enzymes were active with Co^{2+} ions. Incubation with 2 mM Co^{2+} ions gave relative activities of 90% (ppGalNAc-T1), 105% (ppGalNAc-T2), and 50% (ppGalNAc-T6) with reference to standard assay conditions (20 mM Mn^{2+} ions, data not shown). However, the activity of the enzymes was significantly reduced at higher concentrations of Co^{2+} ions (up to 20 mM), which is in contrast to the results obtained for Mn^{2+} ions.[30] A Co^{2+} ion concentration of 0.1 mM was chosen for subsequent transfer experiments. Under these conditions **11** was stable, and the residual activities of the enzymes were 74% (ppGalNAc-T1), 62% (ppGalNAc-T2), and 29% (ppGalNAc-T6) (data not shown).

The acceptor in the ppGalNAc-T experiments was the synthetic peptide TAP 25 with the sequence of one tandem repeat and five further amino acids of the cancer-associated mucin MUC1 (peptide sequence: *T*APPAHGV*TS*APD*T*RPAPG*ST*APPA). The peptide has six potential *O*-glycosylation sites (italicized). It is known from previous examinations by mass spectrometry that ppGalNAc-T1 and ppGalNAc-T3 transfer α-D-GalNAc onto three of these sites and GalNAc-T2 transfers additionally onto a fourth site.[30] The latter site is only occupied when the transfer onto the other sites is completed. GalNAc-T1, -T2, and -T6 were tested with **11**, and analysis of these transfer experiments by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) showed that **11** was not transferred (data not shown). These results suggest that the amidocaproylbiotin moiety of the donor substrate **11** is too bulky to be transferred by the tested ppGalNAcTs. Our results were confirmed by a recent report that a 6-deoxy analogue of **2** was not accepted by the recombinant bovine ppGalNAc-T1.[67] Work is in progress in our laboratory to synthesize a derivative of **2** carrying a short chain functional group for subsequent chemoselective coupling to a label or tag.

Conclusion

The preparative chemoenzymatic syntheses of UDP-6-biotinyl-Gal(NAc) compounds **10** and **11** demonstrate the possibility of modifying nucleotide sugars with high yields. The combination of an enzymatic and chemical reaction in one pot led to a very efficient synthesis which is also adaptable for the production of other derivatives of UDP-Gal(NAc). Although the transfer of UDP-6-biotinyl-GalNAc (**11**) by GalNAc-Ts has yet to be demonstrated, the preliminary results for a transfer with UDP-6-biotinyl-Gal (**10**) by β4Gal-Ts and β3Gal-T5 are promising for a glycosyltransferase-mediated tagging of glycoconjugates. This technique may be exploited for the analysis of "under-galactosylated" glycans of glycoproteins or glycolipids related to certain diseases. Also the production process of therapeutic glycoproteins could be monitored for incomplete galactosylation.

Experimental Section

General:

^1H one and two dimensional NMR spectra were recorded on a Bruker AMX-500 or Bruker AMX-600 instrument. ^{13}C NMR spectra were recorded on a Bruker AC-300 instrument. Fast atom bombardment (FAB) MS of **10** was recorded on a JEOL JMS-SX/SX 102A instrument (Jeol, Tokyo, Japan). MALDI-TOF MS of **11** was recorded in negative ion mode on a Voyager-DE instrument (Perseptive Biosystems Inc., Framingham, MA, USA). The processing of the data was performed with GRAMS/386 software (Galactic Industries Corporation).

Galactose oxidase was purchased from ICN (Eschwege, Germany). Bovine β4-galactosyltransferase 1 (recombinant) was purchased from Calbiochem (Bad Soden, Germany). All other recombinant glycosyltransferases were produced and purified as described previous-

ly.[16, 21, 24, 30, 32] UDP-Gal (**1**) and UDP-GalNAc (**2**) were synthesized as described previously.[68–70] Recombinant human β4Gal-T1 was expressed as soluble protein in *E. coli* and purified to homogeneity (Elling, Schumacher, Klein, and Freudl, unpublished results). BSA–$(GlcNAc)_{17}$ was purchased from Sigma (Deisenhofen, Germany) and ovalbumin from Calbiochem (Bad Soden, Germany). All other commercial enzymes were purchased from Roche Diagnostics (Mannheim, Germany). One unit (U) of enzyme is the amount which produces a change of 1.0 extinction unit at 405 nm. All chemicals, unless otherwise stated, were purchased from Sigma (Deisenhofen, Germany).

HPLC analysis of UDP-Gal(NAc) derivatives:

Nucleosides, nucleoside mono- and diphosphates, nucleotide sugars, and biotinylated nucleotide sugars were analyzed by ion-pair reversed-phase HPLC according to Elling and Kula,[71] with a methanol concentration of 20%. The 6-aldo nucleotide sugars **3** and **4** eluted at 3.96 and 3.70 min, respectively. The 6-biotin-hydrazono nucleotide sugars **7** and **8** eluted at 31.0 and 33.27 min, respectively. The 6-biotinyl nucleotide sugars **10** and **11** eluted at 23.19 and 23.10 min, respectively. With 5% methanol in the elution buffer, the uronic acids **5** and **6** eluted at 40.0 and 39.97 min, respectively.

Optimized conditions for the stepwise synthesis of UDP-6-biotin-hydrazono-Gal (7):

The enzymatic oxidation of **1** was performed in HPLC sample vessels (1-mL volume) that were closed by a septum and equipped with a tube. A balloon filled with oxygen was fixed to the tube to supply an oxygen atmosphere for the reaction of galactose oxidase. Catalase (13000 U mL^{-1}) was added to a solution of **1** (8 mM) in buffer A (25 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0)) containing 0.5 mM $CuSO_4 \cdot x\,H_2O$. The reaction was started by the addition of galactose oxidase (15 U mL^{-1}). The final volume was 250 μL. Samples of 10 μL were taken at the indicated incubation periods and heated for 5 min at 95 °C. Denatured proteins were removed by centrifugation (15 min, 12000 rpm, Eppendorf centrifuge), and the supernatant was analyzed by HPLC for the formation of **3** and **5**. The enzyme reaction was terminated after 4 h incubation as described above. Chemical coupling was performed by the addition of biotin-ε-amidocaproyl-hydrazide (**9**; 1.5 equiv) to the protein-free reaction solution. After a 2 h incubation the reaction mixture was analyzed by HPLC for the formation of **7**.

Optimized conditions for the chemical reduction of UDP-6-biotin-hydrazono-Gal (7):

Batches (100 μL) from the synthesis of **7** (see above) were cooled to 0 °C. $NaCNBH_3$ (0.1 mg μL^{-1}) was dissolved in ice-cold buffer A immediately before use and 5 μL of this solution, corresponding to 30 equiv of **7**, were added. Each reaction batch (105 μL) was distributed over ten reaction vials, which were incubated at different temperatures as indicated. Each sample was analyzed by HPLC for the formation of the product **10** and the decomposition product **3**. The yields obtained by HPLC analysis refer to the sum of the relative peak areas (100%) for **7**, **10**, and **3**.

One-pot chemoenzymatic synthesis of UDP-6-biotinyl-Gal (10):

The preparative synthesis was performed in a thermostated 50-mL reactor with an integrated bubble-free aeration system. Compound **9** (180 mg, 12 mM) was dissolved in buffer A at 30 °C. The temperature was reduced to 15 °C and UDP-Gal (**1**; 210 mg, 8 mM), $CuSO_4$ (0.5 mM), and catalase (6500 U mL^{-1}) were added. The synthesis was started by the addition of galactose oxidase (3 U mL^{-1}). The final volume of the reaction was 40 mL. After 56 h the temperature was reduced to 4 °C and the enzymes were separated from the crude product solution by ultrafiltration in a stirred ultrafiltration cell, model 8050 equipped with a YM30 membrane (Amicon, Witten, Germany). The product solution was cooled to 0 °C. $NaCNBH_3$ (10 equiv, 251 mg) dissolved in ice-cold buffer A (2.5 mL) was added and the reaction solution was incubated for 48 h at −20 °C in a freezer. After isolation by preparative HPLC, gel filtration, and lyophilization, an overall yield of 38% (128 mg) of **10** was obtained. FAB-MS: *m/z* found: 942.05 $[M+H]^+$, calculated: 942.3 (X_1 = Na, X_2 = H); ^{1}H NMR (500 MHz, D_2O, 300 K): δ = 7.957 (U-H_6), 5.978 (R-H_1), 5.966 (U-H_5), 5.641 ($^3J_{1,2}$ = 3.5, $^3J_{1,P}$ = 6.5 Hz, A-H_1), 4.599 (B-H_4), 4.414 (B-H_3), 4.366 (R-H_2), 4.360 (R-H_3), 4.279 (R-H_4), 4.221 (R-H_{5a}), 4.194 (R-H_{5b}), 4.118 (A-H_5), 3.951 (A-H_4), 3.914 (A-H_3), 3.787 (A-H_2), 3.322 (B-H_2), 3.171 (B-H_ε), 3.050 (A-H_{6a}), 2.999 (B-H_{5a}), 2.995 (A-H_{6b}), 2.782 (B-H_{5b}), 2.254 (B-H_α), 2.220 (B-H_ξ), 1.716 (B-H_δ), 1.569 (B-H_β), 1.569 (B-H_δ), 1.508 (B-H_η), 1.403 (B-H_γ), 1.300 (B-H_η).

One-pot chemoenzymatic synthesis of UDP-6-biotinyl-GalNAc (11):

The preparative synthesis was performed in a thermostated 50-mL reactor with integrated bubble-free aeration system. Compound **9** (126 mg, 12 mM) was dissolved in buffer A at 30 °C. The temperature was reduced to 15 °C and UDP-GalNAc (**2**; 150 mg, 8 mM), $CuSO_4$ (0.5 mM), and catalase (6500 U mL^{-1}) were added. The synthesis was started by addition of galactose oxidase (12 U mL^{-1}). The final volume of the reaction was 30 mL. After 65 h the temperature was reduced to 4 °C and the enzymes were separated from the product solution by ultrafiltration in a stirred ultrafiltration cell as described above. The product solution was cooled to 0 °C. $NaCNBH_3$ (10 equiv, 141 mg) dissolved in ice-cold buffer A (1.4 mL) was added and the reaction solution was incubated for 48 h at −20 °C. After isolation by preparative HPLC, gel filtration, and lyophilization, an overall yield of 40% (98 mg) of **11** was obtained. MALDI-TOF-MS: *m/z* found: 959.331 $[M-H]^-$, calculated: 959.3 ($X_{1,2}$ = H); ^{1}H NMR (500 MHz, D_2O, 300 K): δ = 7.956 (U-H_6), 5.978 (R-H_1), 5.962 (U-H_5), 5.562 ($^3J_{1,2}$ = 3.5, $^3J_{1,P}$ = 6.5 Hz, A-H_1), 4.612 (B-H_4), 4.426 (B-H_3), 4.369 (R-H_2), 4.356 (R-H_3), 4.293 (A-H_5), 4.287 (R-H_4), 4.250 (R-H_{5a}), 4.241 (A-H_2), 4.194 (R-H_{5b}), 3.953 (A-H_3), 3.926 (A-H_4), 3.334 (B-H_2), 3.169 (B-H_ε), 3.077 (A-H_{6a}), 3.007 (A-H_{6b}), 2.997 (B-H_{5a}), 2.779 (B-H_{5b}), 2.252 (B-H_α), 2.215 (B-H_ξ), 2.079 (A-H_{Ac}),1.724 (B-H_δ), 1.634 (B-H_β), 1.581 (B-H_δ), 1.506 (B-H_η), 1.404 (B-H_γ), 1.318 (B-H_η).

Enzymatic synthesis of UDP-GalA (5):

The synthesis of **5** was performed in a thermostated 20-mL reactor with integrated bubble-free aeration. UDP-Gal (**1**; 72.3 mg, 12 mM) was dissolved in buffer A containing 0.5 mM $CuSO_4$. The solution was saturated with oxygen at 15 °C. Galactose oxidase (10 U mL^{-1}) and catalase (6500 U mL^{-1}) were added to a final volume of 10 mL. After 20 h of incubation the enzymes were separated from the product solution by ultrafiltration as described above. After isolation by preparative HPLC, gel filtration, and lyophilization, an overall yield of 8.3% (6 mg) of **5** was obtained. ^{1}H NMR (600 MHz, D_2O, 300 K): δ = 7.927 (d, 1 H, $^3J_{5'',6'}$ = 8.4 Hz, U-H6), 5.968 (d, 1 H, $^3J_{1',2'}$ = 4.2 Hz, R-H_1), 5.958 (d, 1 H, $^3J_{5'',6'}$ = 8.4 Hz, U-H_5), 5.730 (m, 1 H, A-H_1), 4.473 (m, 1 H, A-H_5), 4.342 (m, 2 H, R-H_2, R-H_3), 4.295 (d, 1 H, $^3J_{4,5}$ = 2.4 Hz, A-H_4), 4.256 (m, 1 H, R-H_4), 4.193 (d, 1 H, $^2J_{5a,5b}$ = 11.4 Hz, R-H_{5a}), 4.138 (d, 1 H, $^2J_{5a,5b}$ = 10.2 Hz, R-H_{5b}), 3.967 (dd, 1 H, $^3J_{2,3}$ = 10.2 Hz, $^3J_{3,4}$ = 3.0 Hz, A-H_3), 3.842 (d, 1 H, $^3J_{2,3}$ = 8.4 Hz, A-H_2); ^{13}C NMR (75 MHz, D_2O, 300 K): δ = 175.53 (A-C_6), 166.48 (U-C_4), 152.11 (U-C_2), 141.85 (U-C_6), 102.96 (U-C_5), 96.12 (d, $^2J_{1,P}$ = 6.69 Hz, A-C_1), 88.51 (R-C_1), 83.65 (R-C_4), 74.03 (R-C_2), 73.12 (A-C_5), 70.88 (A-C_3); 69.92 (R-C_3); 69.72 (A-C_4) 68.39 (A-C_3), 65.17 (R-C_5).

Isolation of UDP-GalA (5), UDP-6-biotinyl-Gal (10), and UDP-6-biotinyl-GalNAc (11):

The compounds **5**, **10**, and **11** were isolated by preparative HPLC using a reverse-phase C18 HPLC column (ODS-Hypersil, 10 μm, 250 × 10 mm (Gynkotek, Germering, Germany)). The elution was isocratic at a flow-rate of 10 mL min^{-1} with 0.1 M potassium acetate, adjusted by acetic acid to pH 5.6, and containing 20% methanol (5% for the isolation of **5**) and 0.013% *n*-octylamine. The pooled fractions of compounds **5**, **10**, and **11**, individually, were desalted by gel filtration over Sephadex G-10 (Pharmacia, Freiburg, Germany). Samples were loaded and eluted with distilled water at a flow rate of 1 mL min^{-1} on a XK26/100 column (Pharmacia, Freiburg, Germany) with a bed volume of 493.5-mL gel. The elution of nucleotide sugars and salt was monitored by an UV detector at 254 nm and a conductivity detector cell, respectively. Product-containing fractions were pooled and lyophilized.

Enzyme assays:

Galactose oxidase: Galactose oxidase activity was tested by the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS)[72] with D-galactose, **1**, and **2** as substrates.

Galactosyltransferases: The standard assay[21] for all tested galactosyltransferases was performed in a 50-μL volume reaction mixture containing 50 mM HEPES/NaOH buffer (pH 7.4), 5 mM $MnCl_2$, 0.1% Triton X-100, 90 μM UDP-[^{14}C]Gal (1300 cpm nmol^{-1}; Amersham), and 0.25 mM β-d-GlcNAc-Bz (for β4Gal-T2), 1 mM β-d-GlcNAc-Bz (for bovine β4Gal-T1 and human β4Gal-T3), 2 mM β-d-GlcNAc-Bz (for β3Gal-T5), or 20 mM β-D-GlcNAc-Bz (for β4Gal-T4). Bz = benzoyl.

The galactosyltransferases were also tested as described above in the absence of $MnCl_2$. However, the preparations of β4Gal-T2, β4Gal-T3, and β4Gal-T4 contained 1 mM Mn^{2+} ions and were therefore desalted by ultrafiltration prior to use in activity assays and transfer experiments (see below). The enzyme solutions (200 μL) were concentrated to 50 μL in a microcon 10 (Amicon, Witten, Germany) at 4 °C in a centrifuge (Eppendorf, 13 000 rpm, 15 800 g). After addition of 25 mM tris(hydroxymethylaminomethane (Tris)/HCl buffer (pH 7.5; 300 μL), the solution was concentrated again. This procedure was repeated three times and glycerol was added to the resulting enzyme solution.

The activity of purified human β4Gal-T1 expressed in *E. coli* was assayed in 100 mM HEPES/NaOH buffer (pH 8.0) containing 1 mM $MnCl_2$, 2.5 mM UDP-Gal, and 25 mM D-GlcNAc, and was analyzed by HPLC as described previously.[73] The activity was also tested without Mn^{2+} ions in the buffer.

Polypeptide *N*-acetylgalactosaminyltransferases: The standard assay[30] for the polypeptide GalNAc-Ts (ppGalNAc-T1, ppGalNAc-T2, and ppGalNAc-T6) was performed in a 50-μL volume reaction mixture containing 25 mM cacodylate buffer (pH 7.4), 20 mM $MnCl_2$, 0.1% Triton X-100, 50 μM UDP-[^{14}C]GalNAc (4000 cpm nmol^{-1}; Amersham) and 0.1 mM TAP 25 (peptide sequence: *T*APPAHGV*TS*APD*T*RPAPG*ST*APPA). The italicized amino acids indicate the potential glycosylation sites in the peptide. Enzyme activities were also tested with different $CoCl_2$ concentrations (0 – 20 mM) instead of $MnCl_2$.

Transfer reactions with galactosyltransferases and GlcNAc-MU as the acceptor:

The transfer reactions with the Mn^{2+}-free galactosyltransferases (see above) were performed as follows: β3Gal-T5 (0.6 mU mL^{-1}), β4Gal-T4 (1.2 mU mL^{-1}) or β4Gal-T3, -T2, or -T1 (2 mU mL^{-1} of each) were incubated for 16 h (for donor substrate **1**) or 19 h (for donor substrate **10**) at 37 °C in 25 mM HEPES/NaOH buffer (pH 7.4) containing donor substrate **1** or **10** (5 mM), GlcNAc-MU (2 mM for β3Gal-T5; 20 mM for β4Gal-T4; 1 mM for β4Gal-T3 and -T1; 0.25 mM for β4Gal-T2), and 0.1% Triton X-100 in a final volume of 70 μL. In the experiment with β3Gal-T5 0.3 mU mL^{-1} of the enzyme were added after 16 h and incubated for 3 h at 37 °C. Aliquots were taken after 0, 16, and 19 h and analyzed by TLC and HPLC. TLC was conducted on Baker Si_{250F} silica gel (50 μm) TLC plates with a fluorescence indicator (Baker, Philippsburg, USA) as described previously.[74] In the first run the solvent mixture A (acetone/ethylendiamine/water (60:40:1)) was used for the separation of detergent. A second run in solvent mixture B (chloroform/methanol/water (50:40:10)) for 20 min followed. The acceptor GlcNAc-MU and the transfer products were visible by irradiation at 366 nm. The nucleotide sugars were visible by irradiation at 254 nm. GlcNAc-MU: $R_f = 0.87$, UDP-Gal: $R_f = 0.08$, UDP-6-biotinyl-Gal: $R_f = 0.08$, transfer products: $R_f = 0.71$.

HPLC analysis was conducted on a Glycosep N column (5 μm, 250 mm × 4 mm (OGS, Oxford, UK)). In addition to UV detection at 254 nm, a fluorescence detector was used (excitation: 365 nm, emission: 450 nm). Separation of oligosaccharides was achieved by an acetonitrile gradient in 0.1 M ammonium formate (pH 4.4; adjusted by formic acid) at room temperature.

Transfer reactions with galactosyltransferases and BSA – $(GlcNAc)_{17}$ and ovalbumin as the acceptors:

The neoglycoconjugate BSA – $(GlcNAc)_{17}$ carrying 17 mol of GlcNAc per mol of BSA (average molecular mass: 70 kD) and ovalbumin were used for transfer experiments with human β3Gal-T5, human β4Gal-T1, and human β4Gal-T4. BSA – $(GlcNAc)_{17}$ (1.64 mg) or ovalbumin (1.64 mg) was dissolved in 25 mM HEPES/NaOH buffer (pH 7.4) containing 5 mM **1** or **10**, and 0.1% Triton X-100. The reactions were started by the addition of 0.6 mU mL^{-1} of the enzymes to a final volume of 200 μL. With BSA – $(GlcNAc)_{17}$ the overall concentration of GlcNAc residues on the neoglycoconjugate was 2 mM. β3Gal-T5 (0.3 mU mL^{-1}) was added to BSA – $(GlcNAc)_{17}$ after incubation for 16 h at 37 °C and the reaction mixture was incubated for further 3 h. All experiments with ovalbumin were incubated for 14 h at 37 °C.

The reactions were stopped by removal of the donor substrate by ultrafiltration in a microcon 10 (Amicon, Witten, Germany). Control experiments without enzyme and acceptor substrate, respectively, were performed as described above.

The conversion of **10** was determined by HPLC analysis and compared to a control experiment lacking the enzyme for the experiment with β3Gal-T5 using BSA – $(GlcNAc)_{17}$ as the acceptor.

SDS-PAGE and Western blot analyses of proteins:

Samples of BSA – $(GlcNAc)_{17}$ from transfer experiments with galactosyltransferases were separated by SDS-PAGE with 10% gels according to Schägger.[75] A reference gel of the samples and Comassie-prestained standard proteins (BioRad, München, Germany) was silver-stained according to Blum et al.[76] Samples of ovalbumin from transfer experiments with galactosyltransferases were separated by SDS-PAGE with 10% NuPAGE Bis-Tris gels as described by Invitrogen (Groningen, The Netherlands). A reference gel of the samples and biotinylated standard proteins (BioRad, München, Germany) was silver-stained as described by Blum et al.[76]

The proteins from a parallel gel were transferred onto poly(vinylidene difluoride) (PVDF) Immobilon P membranes (pore size = 0.45 μm; Millipore, Bedford, USA) by electro-blotting according to Kyhse-Andersen.[77] with a constant current of 1.5 mA per mm^2 of gel and a constant voltage of 40 V. After blocking of the membrane with 4% casein in TBS-1 buffer (10 mM Tris/HCl (pH 7.5) with 0.9% NaCl) for 1 h, the blotted proteins were incubated for 1 h with streptavidin – alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) diluted in TBS-1 buffer (1:2500). The membrane was washed twice with TBS-1 buffer for 30 min and equilibrated for 2 min in TBS-2 buffer (100 mM Tris/HCl (pH 9.5) with 100 mM NaCl and 5 mM

$MgCl_2 \cdot 7H_2O$). The enzyme reaction was started by the addition of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/4-nitro blue tetrazolium chloride (NBT) (0.38 mM and 0.40 mM, respectively) in TBS-2 buffer, and it was stopped by the addition of Tris/ethylenediaminetetraacetate (EDTA) buffer (100 mM Tris/HCl (pH 6.5) and 100 mM EDTA) after the dye precipitate had been developed.

Enzyme-linked streptavidin assay (ELSA):

Samples of glycoproteins with the Gal-biotin tag and controls were diluted with TBS-Tween buffer (50 mM Tris/HCl (pH 7.6) with 150 mM NaCl and 0.1% Tween 20) in the range of 1:100 – 1:256 000 corresponding to protein concentrations between 82 $\mu g\,mL^{-1}$ and 32 $ng\,mL^{-1}$. The diluted samples (100 μL) were placed for protein adsorption into the wells of a microtiter plate (Nunc-Immuno module, MaxiSorp 16, Nunc, Wiesbaden, Germany) and incubated overnight at room temperature. After washing all wells twice with TBS-Tween buffer (400 μL), the microtiter plate was blocked for 1 h at room temperature with a gelatine blocking solution for enzyme-linked immunosorbent assays (400 μL; Roche Diagnostics, Mannheim, Germany). After washing, the wells were incubated with of streptavidin – horseradish peroxidase conjugate (100 μL; diluted 1:10 000 in TBS-Tween) for 2 h at room temperature. The plates were washed and enzyme substrate solution (100 μL) containing 0.05% (w/v) *o*-phenyldiamine (OPD) and 0.01% (v/v) H_2O_2 in 0.1 M citric acid/sodium phosphate buffer (pH 5.0; Dako Diagnostika, Hamburg, Germany), was incubated for 2 min (for BSA-$(GlcNAc)_{17}$ with the Gal-biotin tag) or 30 min (ovalbumin with the Gal-biotin tag). After the enzyme reaction was stopped by addition of 0.5 M H_2SO_4 (100 μL), the optical density of each well was measured at 490 nm in a microtiter plate reader (Thermomax, MWG-Biotech, Ebersberg, Germany). All presented data are mean values from triple measurements.

Transfer reactions with polypeptide *N*-acetylgalactosaminyltransferases:

The transfer reactions with the ppGalNAc-Ts were performed as follows: GalNAc-T1 (10 $mU\,mL^{-1}$), GalNAc-T2 (38 $mU\,mL^{-1}$), or GalNAc-T6 (20 $mU\,mL^{-1}$) were incubated for 8 h at 37 °C in 25 mM cacodylate buffer (pH 7.4) containing 2.5 mM **11**, 0.1 mM $CoCl_2$, TAP 25 (500 $\mu g\,mL^{-1}$) and 0.1% CF54 in a final volume of 25 μL. Aliquots of 1 μL were taken after 0 and 8 h and analyzed by MALDI-TOF MS as described previously.[30] Prior to analysis all samples were separated from detergents and salts by the nanocolumn technique.[78] The mass spectra of peptides were recorded in the linear mode.

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