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Combination of UDP-Glc(NAc) 4′-Epimerase and Galactose Oxidase in a One-Pot Synthesis of Biotinylated Nucleotide Sugars

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Abstract: The enzymatic epimerization of uridine 5′-diphospho-α-D-glucose (UDP-Glc, **1**) and uridine 5′-diphospho-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc, **2**) and the subsequent oxidation of uridine 5′-diphospho-α-D-galactose (UDP-Gal, **3**) and uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine (UDP-GalNAc, **4**) were combined with chemical biotinylation with biotin-ε-amidocaproylhydrazide in a one-pot synthesis. Analysis by CE and NMR revealed a mixture (1.0:1.4) of the biotinylated nucleotide sugars uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-galactose (UDP-6-biotinyl-Gal, **7)** and uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-glucose (UDP-6-biotinyl-Glc, **9**), respectively, in a reaction started with **1**. One product, uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-*N*-acetyl-α-D-galactosamine (UDP-6-biotinyl-GalNAc, **8)** was formed when the reaction was initiated with **2**. It could be demonstrated for the first time that a UDP-Glc(NAc) 4′-epimerase (Gne from *Campylobacter jejuni*) and galactose oxidase from *Dactylium dendroides* can be used simultaneously in enzymatic catalysis. This is of particular interest since the coaction of an enzyme demanding reductive conditions and an oxygen-dependent oxidase is unexpected.

Keywords: carbohydrates; enzyme catalysis; glycoconjugates; glycosyltransferases; oxidation

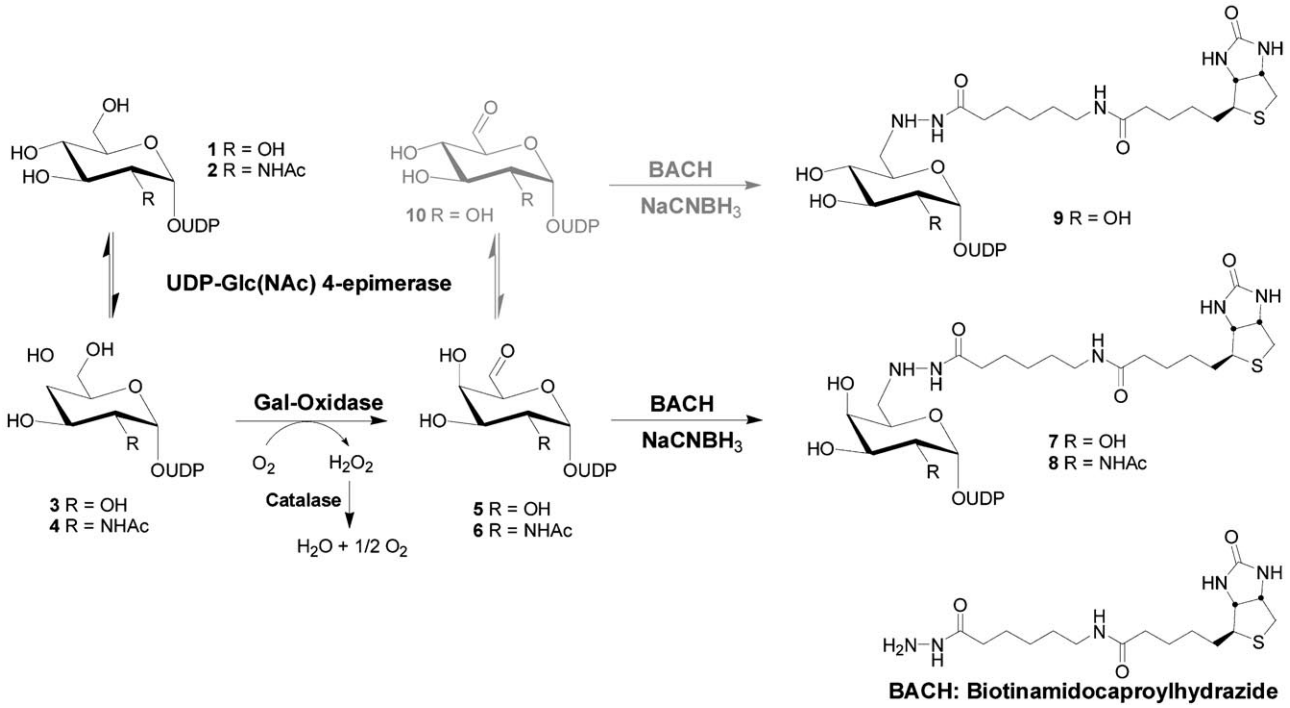
Glycoconjugates play an essential role in biological processes. They are involved in biological recognition,[1] acute and chronic diseases (such as inflammation),[2a,b] and numerous human cancer types.[3a,b] Therefore, techniques opening the possibility for the selective labeling of the relevant glycoproteins and glycolipids are of high interest. These techniques may enable the tracing of aberrant glycosylation in diseases or the design of glycoconjugates for biological recognition and pharmaceutical therapy. The selective labeling of glycoconjugates has been approached in two different ways in the past. The first is an *in vivo* methodology exploiting the acceptance of biosynthetic pathways for chemically modified precursor structures. Metabolic engineering was demonstrated with the sialic acid pathway which was chosen since it shows a high degree of promiscuity.[4a,b,5a–c] In recent work, the salvage pathway for UDP-GalNAc und UDP-GlcNAc was also exploited for metabolic engineering.[6,7] The second approach is based on the design of a nucleotide sugar bearing a chemically introduced label or tag. The modified nucleotide sugar is then transferred by a glycosyltransferase to the glycoconjugate. Again, this *in vitro* strategy was first shown to be successful with sialyltransferases and fucosyltransferases. A fluorometric assay for sialyltransferases was developed on the basis of a chemically modified sialic acid.[8a,b] Also, analogues of GDP-β-L-fucose were shown to be substrates for fucosyltransferases.[9a,b]

Modified donor substrates for the galactosyl- and *N*-acetylgalactosaminyltransferase families have a great potential as tools for labeling under-galactosylated *N*- and *O*-linked glycans in inflammatory diseases such as rheumatoid arthritis or IgA nephropathy.[10a,b,11a,b] In this context, our previous paper described the synthesis of uridine 5′-diphospho-6-biotin-

ε-amidocaproylhydrazino-α-D-galactose (UDP-6-biotinyl-Gal, **7**) and uridine 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-*N*-acetyl-α-D-galactosamine (UDP-6-biotinyl-GalNAc, **8**).[12] We used a straightforward chemo-enzymatic one-pot strategy starting from UDP-Gal **3** and UDP-GalNAc **4**, respectively, in which we combined the oxidation at the C-6 position by the commercial enzyme galactose oxidase from *Dactylium dendroides* (EC 1.1.3.9) (6-aldo intermediates **5** and **6**) with chemical biotinylation using biotin-ε-amidocaproylhydrazide (BACH). A subsequent reduction step, at the 100-mg scale, with $NaCNBH_3$ at −20 °C yielded UDP-6-biotinyl-Gal(NAc) with overall yields between 38 % and 40 %.

The otherwise advantageous synthesis is, however, still hampered by the relative high costs for the nucleotide sugars **3** and **4**. The synthesis of **7** and **8** from the 4′-epimeric nucleotide sugars **1** and **2**, which are commercially available on a large scale,[13] would significantly reduce the costs by a factor of up to 70-fold and require the use of a UDP-Glc(NAc) 4′-epimerase (EC 5.1.3.2 and 5.1.3.7). In the present paper, we followed the chemo-enzymatic one-pot synthesis route outlined in Scheme 1. However; it also depicts the unexpected formation of **9** as demonstrated by CE and NMR analysis. The existing literature suggests that our approach to combine a UDP-Glc(NAc) 4′-epimerase and a galactose oxidase in a one-pot synthesis appeared impractical since UDP-Glc 4′-epimerases need reducing reagents such as dithiothreitol or β-mercaptoethanol for long-term stability,[14] whereas galactose oxidase is irreversibly inactivated by sulfhydryl reagents.[15]

Through the characterization of two recombinant UDP-Glc 4′-epimerases we present here a practical solution for the combination with galactose oxidase. The UDP-Glc 4′-epimerase of *Saccharomyces cerevisiae* has already been used several times in the synthesis of glycoconjugates on a preparative scale.[16a–c] The gene *gal10* encoding the bifunctional enzyme UDP-Glc 4′-epimerase/galactose mutarotase[17a,b] was cloned into an *E. coli* expression vector. The recombinant enzyme was expressed as soluble protein (1 U/mg) and conveniently purified *via* its His_6-Tag (see Supporting Information). The bifunctional UDP-Glc-(NAc) 4′-epimerase (Gne) from *Campylobacter jejuni* was cloned in *E. coli* and characterized previously.[18] The characteristics of this enzyme appeared ideal for our synthesis approach; not only does it epimerize both nucleotide sugars **1** and **2**, but it has by far the highest activity (50 U/mg for both nucleotide sugars) of known bacterial UDP-Glc(NAc) 4′-epimerases. Since Gne is expressed as an MalE fusion-protein, convenient one-step purification easily ensures sufficient purity for biocatalytic applications. We also cloned the GALE gene encoding the bifunctional human UDP-Glc(NAc) 4′-epimerase, but in our hands the expression in *E. coli* resulted in the almost quantitative formation of inclusion bodies (data not shown). Finally, the commercially available UDP-Glc



Scheme 1. Combination of galactose oxidase and UDP-Glc(NAc) 4′-epimerase for the synthesis of biotinylated nucleotide sugars.

4′-epimerase from *Streptococcus thermophilus*, with 10 U/mg specific activity, is prohibitively expensive to be used for preparative synthesis.

The activities of the epimerases from *S. cerevisiae* (Gal10) and *C. jejuni* (Gne) were tested in the absence of mercaptoethanol for 48 h, which is the reaction time for our previous synthesis. We observed a drop of activity to 30% and 10% residual activity within 24 h for Gal10 and Gne, respectively (for details see Supporting Information). However, due to its 50-fold higher specific activity, 10% residual activity (5 U/mg) for Gne appeared to be sufficient for preparative synthesis. After 48 h, the epimerase had still 2% (1 U/mg) residual activity. For comparison, the activity of Gal10 dropped to a value of 0.3 U/mg after 24 h.

In our previous work, we demonstrated that aeration in a bubble-free reactor system is essential for the stability of galactose oxidase.[12] An oxygen-saturated buffer could, however, affect the activity of UDP-Glc(NAc) 4′-epimerases. Two vicinal thiols were shown to form a disulfide bridge under oxidizing conditions leading to inactivation of UDP-Glc 4′-epimerase from *Saccharomyces fragilis*.[20a,b] In the bubble-free aeration reactor the activity of Gne from *C. jejuni* decreased to 4.4% after 24 h. Although Gne from *C. jejuni* is used in large excess (900 U), the present synthesis is economic, because UDP-GalNAc is expensive and Gne can be produced in kU yields.

The equilibrium reaction of UDP-Glc(NAc) 4′-epimerases does not favor the formation of the galacto-epimers and coupling to other enzyme reactions, for example, galactosyltransferases or *N*-acetylgalactosaminyltransferases, is necessary for an efficient conversion of UDP-Glc(NAc).[16a–c,18] At equilibrium, 24% of UDP-Glc and 29% of UDP-GlcNAc are converted to UDP-Gal and UDP-GalNAc, respectively, by Gne from *C. jejuni*.[18] In our reaction scheme, oxidation and biotinylation of UDP-Gal(NAc) drive the equilibrium reaction towards effective conversion of **1** and **2** into **3** and **4**. Therefore, a sufficient activity of the epimerase is needed throughout the duration of the reaction. This was proved by the preparative synthesis in a reactor with bubble-free aeration in the absence of mercaptoethanol. Starting from **1** or **2**, synthesis yields of 82% (**7** and **9**) and 77% (**8**) could be reached after 30 h and 40 h, respectively (Figure 1).

After quantitative reduction with $NaCNBH_3$ at −20 °C and purification by HPLC, a mixture (125 mg) of **7** and **9** (revealed by NMR analysis), and the sole product **8** (131 mg) were obtained with overall yields of 34%. The molecular composition of **8** was determined to be $C_{33}H_{54}N_8O_{19}P_2S$ (found: 959.2626; calculated for $M-H^+$: 959.2623) using high resolution ESI-ToF-MS. Subsequent ^1H NMR analysis of **8** revealed both characteristic modifications of the hexose moiety. As expected the inversion at C-4 resulted in a

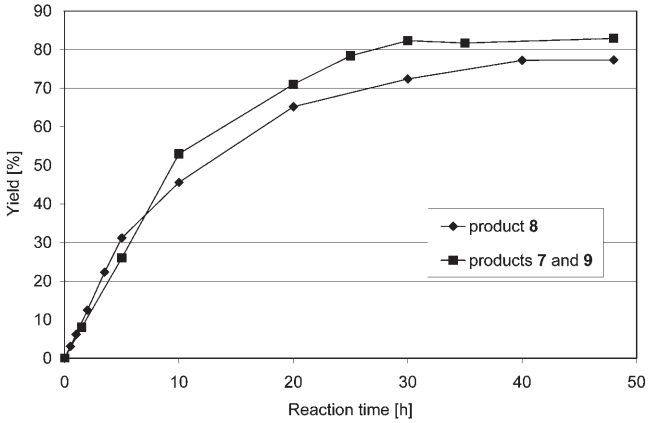


Figure 1. Formation of biotinylated nucleotide sugars detected by HPLC: UDP-6-biotinyl-Gal (**7**) and UDP-6-biotinyl-Glc (**9**) are formed from UDP-Glc (**1**);UDP-6-biotinyl-GalNAc (**8**) is the only product when the reaction was initiated with UDP-GlcNAc (**2**).

singlet for the corresponding proton at 3.84 ppm. Additionally the ^1H and ^{13}C NMR data for the CH_2 group at position 6 (^1H: 2.96 and 2.87 ppm, ^{13}C: 51.47 ppm) nicely fit the predicted values for the acetylated hydrazines.

The ^1H NMR analysis of the product starting from **1** revealed the presence of two different but closely related hexoses in a ratio of 1:1.4. ESI-ToF-MS detected just one species with the molecular composition $C_{31}H_{51}N_7O_{19}P_2S$ (found: 918.2355; calculated for $M-H^+$: 918.2357). Further NMR-analysis and comparison with separately synthesized 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-galactose (**7**) proved that the mixture consists of 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-glucose (**9**) as the main compound together with 5′-diphospho-6-biotin-ε-amidocaproylhydrazino-α-D-galactose (**7**). Besides all ^{13}C NMR data[19] the typical diaxial spin-coupling pattern for glucose could be identified. For further spectral data see the Supporting Information. In addition, subsequent CE analysis revealed two products (**7** and **9**) in a ratio of 1:1.49 for the peak areas. In contrast, CE analysis of the isolated product starting from **2** shows only one product (**8**) peak (see Supporting Information). The question was addressed whether Gne is able to epimerize the pure compound **7**[12] to yield **9**. We found that **7** is not a substrate for the epimerase (see Supporting Information) and conclude that product **9** is most probably formed by the Gne-catalyzed epimerization of **5**, yielding the 6-aldo intermediate **10**, and subsequent chemical biotinylation (Scheme 1, possible reaction pathway depicted in grey). However, the 6-aldo intermediate **6** is obviously not epimerized, which may be due to the slightly higher activity (about 11% considering V_{max}) and better equilibrium ratio (29% compared to 24%) for epimerization of UDP-GlcNAc in contrast to UDP-

Glc.[18] Work is in progress to elucidate conditions for Gne to epimerize **5** and **6**. Formation of **8** *via* the oxidation of **2** and subsequent epimerization can be excluded because of the absolute specificity of galactose oxidase for D-galactose.[21]

We have now reported the first chemoenzymatic one-pot synthesis involving the successful interplay between a UDP-Glc(NAc) 4′-epimerase and galactose oxidase. Additionally, our results also indicate for the first time that Gne is able to epimerize C-6-oxidized UDP-Gal. In conclusion, we present an economical access to biotinylated UDP-Gal(NAc) and biotinylated UDP-Glc as a prerequisite for the development of diagnostic assays of disease-related undergalactosylated *N*- and *O*-glycans in rheumatoid arthritis and IgA nephropathy, which is in progress in our group.

Experimental Section

UDP-Glc and UDP-GlcNAc were from Kyowa Hakko, Japan. Galactose Oxidase was from Worthington Biochem, USA. Catalase was from Merck, Germany. UDP-Glc(NAc) 4′-epimerase was produced and purified as described previously.[18] All reagents were from Sigma-Aldrich. ^{1}H NMR spectra were obtained at 400 MHz, ^{13}C NMR spectra at 100 MHz on a Bruker DPX-400. ESI mass spectra were recorded using an LCT spectrometer (Mircomass) equipped with a lockspray dual-electrospray ion source. A Hypersil ODS column (5 μm) from Thermo Electron, USA, was used for HPLC analysis and product purification. Further experimental data are described in the Supporting Information section.

Chemoenzymatic Synthesis of UDP-6-biotinyl-Gal/ UDP-6-biotinyl-Glc 7/9 and UDP-6-biotinyl-GalNAc 8

The preparative synthesis was performed in a 50-mL reactor with an integrated bubble-free aeration system. The biotinylation reagent BACH (222.9 mg, 0.6 mmol) was dissolved in H_2O (bidest.) by heating to 60 °C. After cooling to room temperature, nucleotide sugar **1** (244 mg, 0,4 mmol) or **2** (261 mg, 0.4 mmol), sodium phosphate buffer (50 mM, pH 6.0) and catalase (60,000 U) were added. The pumping circle was started and an overpressure of 0.1 mbar was applied. When the overpressure was constant for 10 min, galactose oxidase (150 U) and UDP-Glc(NAc) 4′-epimerase from *C. jejuni* (900 U) were added to the reaction solution for a final volume of 50 mL. After 30 h and 40 h, respectively, the enzymes were separated from the crude product solution by ultrafiltration *via* a 10 kDa-membrane (Millipore). The product solution was cooled to 0 °C. $NaCNBH_3$ (754.1 mg, 30 mole equivalents) was added and the reaction solution was incubated for 48 h at −20 °C. The reaction products were isolated by RP-HPLC (mobile phase: 1% methanol in H_2O, pH 7.0). After lyophilization, overall yields of 34% for the mixture of **7** and **9** (125 mg), as well as for **8** (131 mg) were obtained.

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