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Chemo-Enzymatic Synthesis of the Galili Epitope Galα(1→3)Galβ(1→4)GlcNAc on a Homogeneously Soluble PEG Polymer by a Multi-Enzyme System

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Abstract—The α-Gal trisaccharide Galα(1→3)Galβ(1→4)GlcNAc **11** was synthesized on a homogeneously soluble polymeric support (polyethylene glycol, PEG) by use of a multi-enzyme system consisting of β-1,4-galactosyltransferase (EC 2.4.1.38), α-1,3-galactosyltransferase (EC 2.4.1.151), sucrose synthase (EC 2.4.1.13) and UDP-glucose-4-epimerase (EC 5.1.3.2). In addition workup was simplified by use of dia-ultrafiltration. Thus the advantages of classic chemistry/enzymology and solid-phase synthesis could be united in one. Subsequent hydrogenolytic cleavage afforded the free α-Gal trisaccharide.

Oligosaccharides promise to play an important role as drugs in future due to their role in biological recognition processes.[1,2] However, their synthesis is cumbersome and their purification difficult and time consuming. Here, we present a methodology for the synthesis of oligosaccharides on a homogeneously soluble polymeric support. The first step involves the chemical synthesis of a monosaccharide on a soluble polymeric support. Elongation of the saccharide follows by stepwise enzymatic glycosyltransferase reactions. Finally, the product is isolated by aid of diafiltration of the polymer enlarged carbohydrate. A soluble support was chosen in order to enable good accessibility of the carbohydrate substrate to the enzymes. Moreover, by use of a soluble polymer support analytical methods are significantly simplified compared to solid-phase synthesis. This technique, which is applicable both to organic as well as to enzymatic synthesis, is expected to contribute to further large-scale synthesis of oligosaccharides, especially those containing molecular recognition sites. Previously, such an approach using glycosidases in reverse in combination with a polyethylene glycol-supported solution-phase technique was described.[3] However, these transformations suffer from extremely low conversion. Subsequent to the transglycosylation, the polymer (polyethylene glycol, PEG) was codistilled with toluene in order to remove water, redissolved in dichloromethane and then precipitated by use of *tert*-butyl methyl ether, followed by filtration and recrystallization. Use of the more favorable glycosyltransferases was not possible due to the fact that metal ions, necessary for the use of transferases, prevent complete precipitation of PEG.[4]

Here we demonstrate that the application of a polymer-supported solution-phase technique as described for a chemical synthesis by Krepinsky et al.[5] *in combination with ultrafiltration* enables the use of glycosyltransferases yielding quantitative conversion. This was shown in the example of the trisaccharide Galα(1→3)Galβ(1→4)GlcNAc (also referred to as Galili epitope). This α-Gal epitope[6–8] is expressed on xenotissues from pigs and is responsible for the hyperacute rejection in humans.[9–11] The *technique* presented here not only contributes to large-scale synthesis of free α-Gal epitopes, but the product, for example *polymer bound α-Gal trisaccharide* itself, may be a useful compound to prevent hyperacute xenograft rejection, since

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prolonged intravascular retention times are reported.[12] An advantage concerning eventual large-scale production is the extremely low price of PEG as support (approx US$ 18 per kg).

An α-Gal trisaccharide supported by the water soluble support polyacylamide was reported by Wang et al.,[13] where the trisaccharide was first synthesized and then coupled to the polymer. Here we first attached the *N*-acetylglucosamine to the soluble polymer polyethylene glycol and then built up the trisaccharide α-Gal by glycosyltransferases. Purification steps are minimized, analytical methods are significantly simplified and a very defined product is yielded.

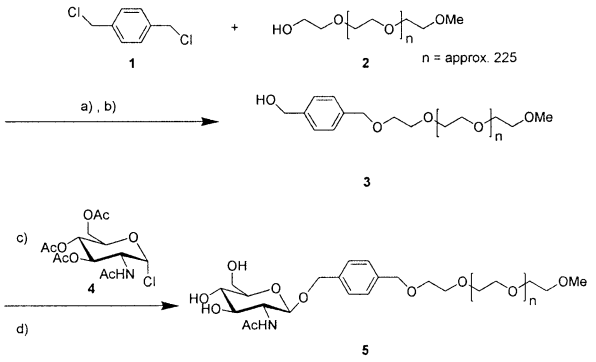

Figure 1. Synthesis of polymer-bound *N*-acetylglucosamine. Reaction conditions: (a) NaH, NaI, THF, rt, 96 h, 97% yield; (b) 10% aqueous Na_2CO_3, 70 °C, 16 h, quant; (c) AgOTf, DCM, ms 4 Å, 50 °C, 16 h, quant; (d) DBU, MeOH, rt, 16 h, quant.

Polyethylene glycol was chosen as support as it has a genuine solubility in water and many organic solvents; however, precipitation can be achieved by an apolar solvent like diethylether.[4,5,14,15] Metal ions or traces of water lead to incomplete precipitation. In the present study an ω-methoxy polyethylene glycol with an average molecular weight of 10,000 g/mol (MPEG) **2** was used, enabling ultrafiltration and HPLC analysis as shown below (Fig. 5). The terminal methoxy functionality was used as internal standard to determine loadings in NMR experiments.

The *N*-acetylglucosamine moiety was linked via a dioxyxylyl linker (DOX) to MPEG **2** as depicted in Figure 1.[15,16] Nearly quantitative loading of the polymer with linker was achieved after strict exclusion of water traces in the ether synthesis[17] of **3**. In the following step, only the β-anomer **5** of the polymer-supported *N*-acetylglucosamine was obtained in quantitative yield. This is of importance for the subsequent enzymatic transformation, since the α-anomer is not accepted.

For synthesis of the desired trisaccharide **8**, a multienzyme system was used,[6,7] consisting of recombinant human β-1,4-galactosyltransferase (β-1,4-GalT), recombinant murine α-1,3-galactosyltransferase[18] (α-1,3-GalT),

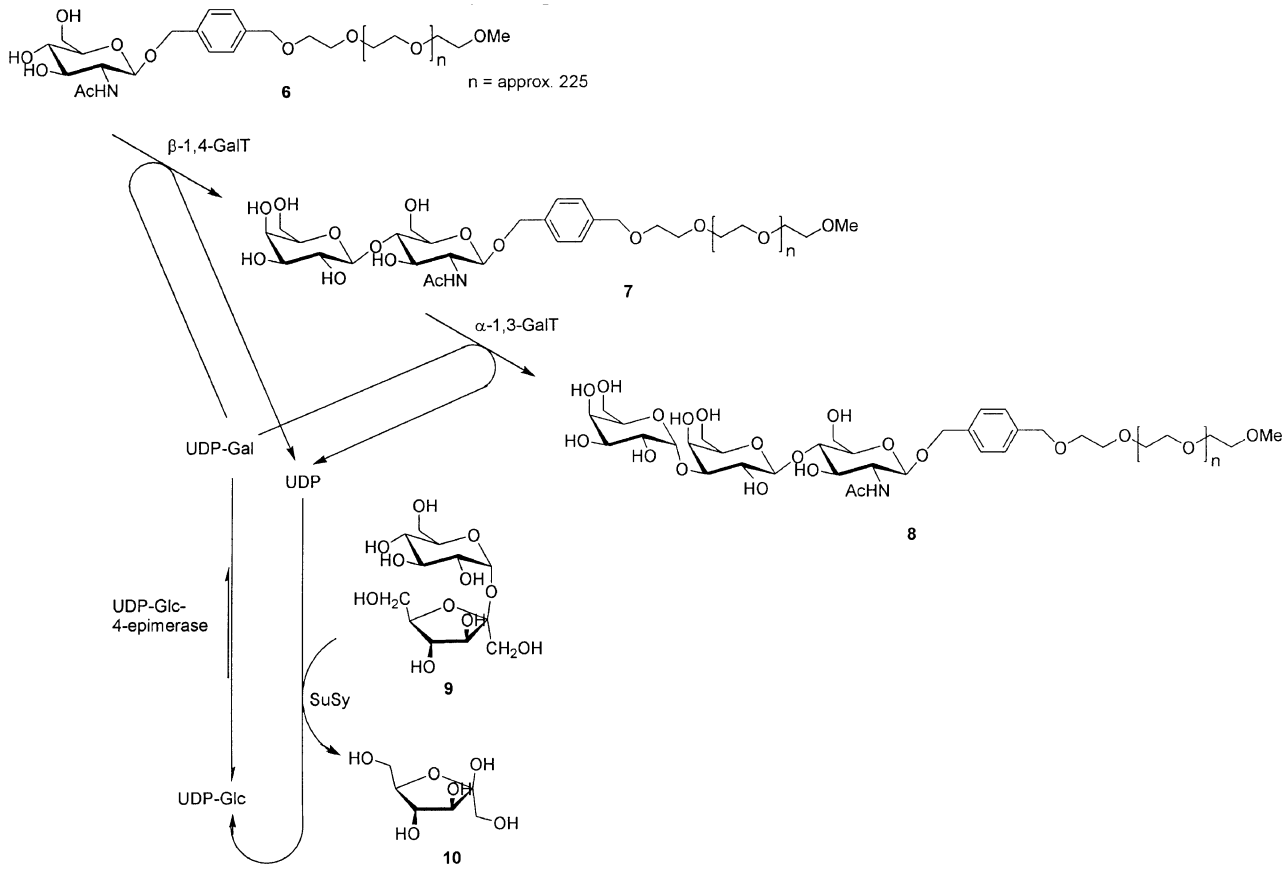

Figure 2. Scheme of the synthesis of polymer-bound Galili trisaccharide **8**. β-1,4-GalT = β-1,4-galactosyltransferase, α-1,3-GalT = α-1,3-galactosyltransferase, SuSy = sucrose synthase, UDP = uridine 5′-diphosphate, UDP-Glc = uridine 5′-diphosphoglucose, UDP-Gal = uridine 5′-diphosphogalactose.

recombinant sucrose synthase[19] (SuSy) from potato and a commercially available recombinant UDP-glucose-4-epimerase from *Streptococcus thermophilus* (Fig. 2). UDP-Gal was (re)generated in situ by combined action of sucrose synthase and UDP-glucose-4-epimerase. The synthesis of the polymer-supported trisaccharide **8** from **6** was started with a catalytic amount of UDP-glucose.[6]

As seen in Figure 3, there is a significant difference in the k_M and k_I values for free and polymer-supported *N*-acetylglucosamine concerning β-1,4-GalT; for data see Table 1. To our knowledge this is the first time that k_M and k_I values of a polymer-supported substrate for a glycosyltransferase were directly measured.

Due to this severe inhibition when using polymer-supported substrate, yields were unsatisfactory using the conditions suited for the free substrate. With the kinetic data in hand it was now possible to run the synthesis in quantitative yields. Best results were obtained starting with a concentration of GlcNAc-DOX-MPEG **6** of 2 mM.

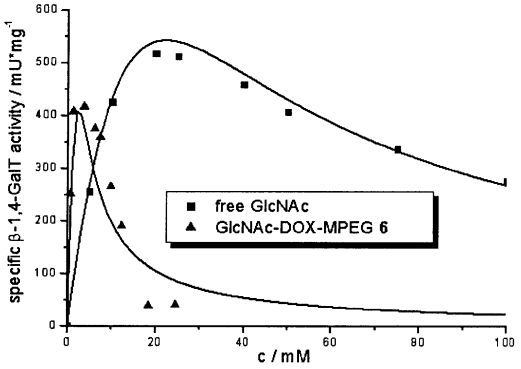

Figure 3. Inhibition of β-1,4-GalT by free and polymer-supported *N*-acetylglucosamine.

Table 1. Kinetic data for β-1,4-GalT, substrate surplus inhibition

	Free *N*-acetylglucosamine	Polymer-supported GlcNAc-DOX-MPEG **6**
k_m (mM)	34.6	10.5
k_I (mM)	14.3	0.54
v_{max} (U/mg)	2.62	4.73

As intermediate product, polymer-supported *N*-acetyllactosamine **7** was formed. Complete conversion was reached after 30 h (Fig. 4), yielding 2.5 g of Galα(1→3)Galβ(1→4)GlcNAc-DOX-MPEG **8**.

Analytical Methods

The course of synthesis was followed by HPLC analysis of the *polymer-supported* carbohydrate derivatives **6**, **7** and **8** (Fig. 5). The corresponding peaks were confirmed by NMR techniques. Thus time consuming NMR measurements for monitoring of polymer-supported reactions[3,20,21] could be replaced. The removal of the polymer was not necessary and a complete baseline separation of the three different polymer-supported carbohydrates **6**, **7** and **8** in the reaction mixture was obtained.

Downstream Processing

Ultrafiltration permitted us to simplify purification of the product. For MPEG **2**, MW 10,000 g/mol, a retention of 0.996 was determined in water using a Pellicon XL 5 kDa module (corresponding to a loss of 0.4% per residence time). Thus, low molecular weight impurities can be washed out by ultrafiltration. Here, precipitated

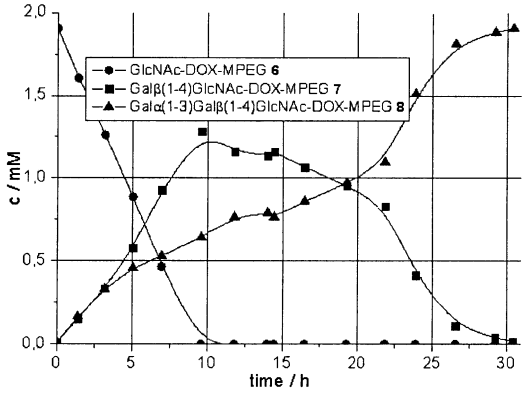

Figure 4. Synthesis of polymer-supported trisaccharide Galα(1→3)Galβ(1→4)GlcNAc-DOX-MPEG **8**. Reaction conditions: $V = 120$ mL, 2 mM **6**, 20 mM HEPES buffer pH 7.2, 25 mM KCl, 1 mM 1,4-dithiothreitol, 200 mM sucrose, 1 mM $MnCl_2$, 0.4 mM UDP-Glc, 1 mg/mL BSA, 4.8 U β-1,4-GalT, 14.4 U α-1,3-GalT, 19.2 U SuSy, 8.6 U UDP-glucose-4-epimerase, $T = 23\,^{\circ}C$, 30 h.

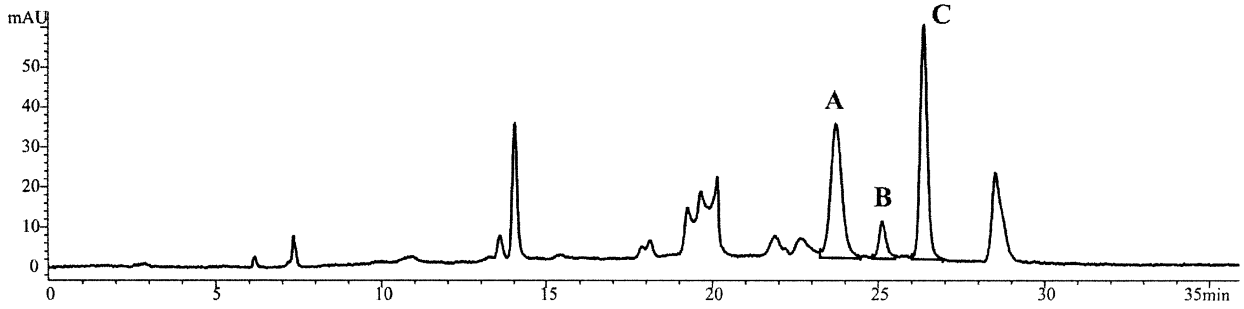

Figure 5. HPLC chromatogram (UV 215 nm) of the three polymer-supported carbohydrates **A** = GlcNAc-DOX-MPEG **6**, **B** = Galβ(1→4)GlcNAc-DOX-MPEG **7**, **C** = Galα(1→3)Galβ(1→4)GlcNAc-DOX-MPEG **8** (reaction mixture, DOX = dioxyxylyl linker, conditions: Nucleodex β-OH column, eluent: ACN–H_2O gradient, 0 min 0% H_2O, 15 min 20% H_2O, 16 min 20% H_2O, 31 min 40% H_2O, 36 min 0% H_2O).

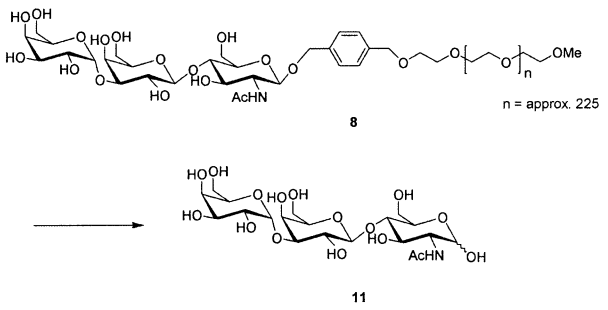

Figure 6. Hydrogenolytic cleavage for liberating the α-Gal epitope **11**. Reaction conditions: 4.0 bar H_2, Pd/C, $HOAc/H_2O = 1:1$, 50 °C, 16 h.

proteins were removed by centrifugation and the product was extracted by DCM. Subsequent dia-ultrafiltration was utilized for further purification. The oligosaccharide was easily cleaved from the polymer by hydrogenation using palladium[5] (Fig. 6). 26 mg of the trisaccharide Galα(1→3)Galβ(1→4)GlcNAc **11** was obtained,[22] meaning an overall yield of 23% starting from compound **6**.

In summary, the presented chemo-enzymatic synthesis of a carbohydrate epitope on a PEG polymer will pave the way to medicinal application, since PEG is a biomaterial generally regarded as safe.

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22. ^1H NMR data (D_2O, 300 MHz, δ in ppm): 5.208 (d, $J_{1,2} = 2.1$ Hz, H-1, GlcNAc, α-anomer); 5.145 (d, $J_{1,2} = 3.8$ Hz, H-1, Gal^3); 4.726 (d, $J_{1,2} = 7.7$ Hz, H-1, GlcNAc, β-anomer); 4.550 (d, 7.7 Hz, H-1, Gal^4); 4.218–4.179 (m, H-5, Gal^3 and H-4, Gal^4); 4.024–3.628 (m, skeleton protons); 2.043 (s, acetyl). A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is linked.