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One-pot enzymatic synthesis of the Galα1→3Galβ1→4GlcNAc sequence with *in situ* UDP-Gal regeneration

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The trisaccharide Galα1→3Galβ1→4GlcNAcβ1→O-$(CH_2)_8COOCH_3$ was enzymatically synthesized, with *in situ* UDP-Gal regeneration. By combination in one pot of only four enzymes, namely, sucrose synthase, UDP-Glc 4′-epimerase, UDP-Gal:GlcNAc β4-galactosyltransferase and UDP-Gal:Galβ1→4GlcNAc α3-galactosyltransferase, Galα1→3Galβ1→4GlcNAcβ1→O-$(CH_2)_8COOCH_3$ was formed in a 2.2 μmol ml^{-1} yield starting from the acceptor GlcNAcβ1→O-$(CH_2)_8COOCH_3$. This is an efficient and convenient method for the synthesis of the Galα1→3Galβ1→4GlcNAc epitope which plays an important role in various biological and immunological processes.

Keywords: α3-galactosyltransferase, sucrose synthase, enzymatic synthesis, UDP-Gal

Introduction

The Galα1→3Galβ1→4GlcNAc sequence is a frequently occurring terminal fragment of the carbohydrate chains of glycoproteins and glycolipids in many species of mammals, with the exception of apes, Old World monkeys and humans [1]. It functions as a ligand in several biological and immunological processes. For example, hyperacute organ rejection in the case of pig to human xenograft transplantation is mainly caused by naturally circulating human antibodies directed against the Galα1 → 3Gal element [2–4]. Furthermore, α3-linked galactose has been implicated in murine sperm-egg binding [5, 6]. In addition, the presence of α3-linked galactose on lactosaminoglycans enhances the binding of galectin-3 (Mac-2) [7].

To be able to further investigate the interaction of α3-galactosylated oligosaccharides with their counter molecules, sufficient amounts of these oligosaccharides have to be available. Specific oligosaccharides often can be synthesized employing glycosyltransferases because of the highly stereo- and regioselective action of these enzymes that usually results in high yields of the desired products. Previously, we have explored the use of recombinant bovine α3-galactosyltransferase (α3-GalT) for *in vitro* enzymatic synthesis of several Galα1→3Gal-containing oligosaccharides [8, 9]. Similarly, large α3-galactosylated oligosaccharides have been synthesized using α3-GalT purified from bovine thymus [10]. However, a drawback of the use of glycosyltransferases is that the availability of the corresponding nucleotide sugars may be limited since these are often hard to synthesize or to isolate and therefore are relatively expensive. One solution for this problem is the application of enzymatic *in situ* regeneration of the nucleotide sugar. For example, a cycle employing five enzymes in which UDP-Gal is generated from Glc 6-phosphate with reutilization of UDP has been devised for the synthesis of Galβ1→4GlcNAc [11, 12]. Recently, we have developed an alternative UDP-Gal regeneration system using sucrose synthase from rice [13]. This enzyme which generates UDP-Glc from sucrose and UDP was used together with β4-GalT and UDP-Glc 4′-epimerase for the synthesis of Galβ1→4GlcNAc.

In the present paper, we show that Galα1→3Galβ1→4GlcNAcβ1→O-R (R = $(CH_2)_8COOCH_3$) can be synthe-

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sized from GlcNAcβ1→O-R in one pot, combining α3-GalT and β4-GalT, and utilizing sucrose synthase and UDP-Glc 4′-epimerase for UDP-Gal regeneration (Fig. 1).

Materials and methods

Materials

Sucrose synthase (EC 2.4.1.13) from rice was purified as described [14, 15]. Prior to use, the sucrose synthase preparation was concentrated ten-fold in 200 mM HEPES, pH 7.2, to a final activity of approximately 10 U ml^{-1}, using an Amicon Centricon 10 concentrator.

Recombinant soluble bovine α3-GalT (EC 2.4.1.151) was produced in *Trichoplusia ni* High-Five insect cells (Invitrogen) by deleting from the 5′ end of a bovine α3-GalT cDNA [16] a portion (81 nucleotides) that encodes the amino-terminal tail and the signal-anchor sequence, prior to ligation into the transfer vector pVT-Bac (kindly donated by Dr T. Vernet, Biotechnology Research Institute, Québec, Canada). The construct was used to generate a recombinant baculovirus. Infection of High Five insect cells in serum-free Excell 401 medium (Seralab) led to the production of soluble α3-GalT, which was purified from the culture medium by affinity chromatography on UDP-hexanolamine Sepharose according to [17]. This procedure yielded a final preparation of 80–150 mU ml^{-1} α3-GalT in 30 mM Tris/maleate, pH 6.5, containing 0.2 mM $MnCl_2$.

SepPak C_{18} cartridges and Prep C_{18} (125 Å) were obtained from Waters. β4-GalT (EC 2.4.1.38) from bovine milk and UDP-Glc 4′-epimerase (EC 5.1.3.2) from yeast were purchased from Sigma. GlcNAcβ1→O-$(CH_2)_8COOCH_3$ was a kind gift of Dr O. Hindsgaul (University of Alberta, Edmonton, Alberta, Canada).

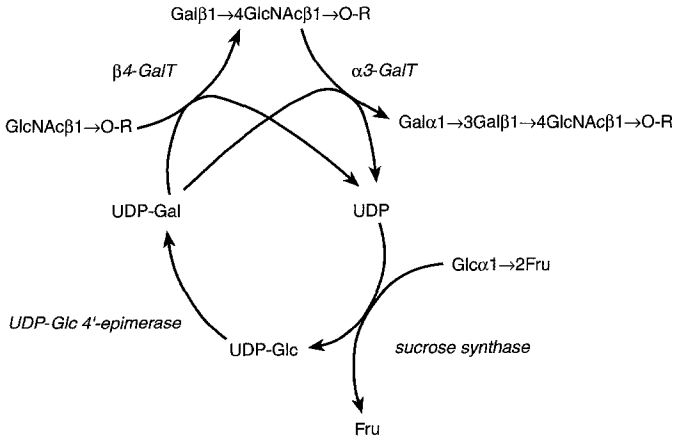

Figure 1. Schematic presentation of the enzymatic synthesis of Galα1→3Galβ1→4GlcNAcβ1→O-R from GlcNAcβ1→O-R with *in situ* UDP-Gal regeneration, using four enzymes: sucrose synthase, UDP-Glc 4′-epimerase, β4-GalT and α3-GalT.

Enzyme activity assays

Sucrose synthase activity was determined by incubating purified sucrose synthase for 10 min at 30 °C in 200 mM HEPES, pH 7.2, containing 500 mM sucrose and 2 mM UDP. Aliquots of the incubation mixtures were analyzed by HPLC on a PartiSphere SAX column (0.5 × 10 cm, Whatman). The column was eluted at a flow rate of 1 ml min^{-1} with 5 mM KH_2PO_4 pH 4.0, applying a gradient by increasing the KH_2PO_4 concentration to 50 mM and the pH to 4.5 in 30 min. The eluate was monitored at 280 nm. The activity of sucrose synthase was calculated from the area of the UDP-Glc peak by comparison with a UDP-Glc standard curve. One U sucrose synthase catalyses the formation of 1 μmol UDP-Glc per min.

The activities of α3-GalT and β4-GalT were assayed as described [17], using lactose and GlcNAc as acceptor, respectively.

Incubations and isolation of the products

With reference to our previously described UDP-Gal *in situ* regeneration system [13], optimized conditions have been applied for the synthesis of Galα1→3Galβ1→4GlcNAcβ1→O-R (Zervosen and Elling, unpublished results). A volume of 5 ml containing 1 mmol HEPES, 2.5 mmol sucrose, 10 μmol $MnCl_2$, 5 μmol UDP-Glc, 0.5 μmol TDP-6-deoxy-4-keto-glucose (to reactivate UDP-Glc 4′-epimerase), 5 mg BSA, 12.5 μmol or 25 μmol GlcNAcβ1→O-R, 2 U sucrose synthase, 1 U UDP-Glc 4′-epimerase, 500 mU β4-GalT and 250 mU α3-GalT was incubated for 88 h at 37 °C and pH 7.2.

To monitor the time course of the product formation, incubations were conducted simultaneously on a 1 ml scale using GlcNAcβ1→O-R concentrations of 2.5 and 5.0 mM, respectively. Aliquots of 5 μl were withdrawn, diluted with 5 ml H_2O and applied to SepPak C_{18} cartridges. After washing with 50 ml of H_2O the products were eluted with 5 ml CH_3OH according to [18]. The isolated products were then analysed by HPEAC with pulsed amperometric detection on a CarboPac PA-1 pellicular anion-exchange column (0.9 × 25 cm, Dionex) as described [9].

Molar ratios of the products were calculated from the corresponding peak areas in the HPEAC chromatograms, taking into account the relative detector response of each compound. The response factor of GlcNAcβ1→O-R was set to 1.0. Response factors of 1.3 for Galβ1→4GlcNAcβ1→O-R and of 1.4 for Galα1→3Galβ1→4GlcNAcβ1→O-R relative to GlcNAcβ1→O-R were determined using identical, ^{3}H-labelled reference compounds of known specific radioactivity. These reference compounds had been enzymatically synthesized using UDP-[^{3}H]Gal as a donor substrate.

For isolation of the products from the 5 ml scale

incubation, the incubation mixture was diluted to 25 ml with H_2O and applied to a column (1 × 7 cm) of Prep C_{18} (125 Å). The column was washed with 500 ml H_2O and eluted with 25 ml CH_3OH at a flow rate of 5 ml min^{-1}. The eluate was dried under a stream of nitrogen, dissolved in 5 ml H_2O and applied to a column (1.6 × 170 cm) of Bio-Gel P-4 (200–400 mesh) which was eluted with 25 mM NH_4HCO_3, pH 7.0, at a flow rate of 37 ml h^{-1}. The eluent was monitored using a refractive index detector. Peak fractions were pooled and carbohydrate-containing fractions (orcinol/H_2SO_4) were analysed by ^{1}H-NMR spectroscopy.

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, samples were exchanged twice in 99.9% ^2H_2O, with intermediate lyophilization. Finally, samples were dissolved in 99.95% ^2H_2O (Merck). ^{1}H-NMR spectra were recorded at 400 MHz on a Bruker MSL-400 spectrometer (Department of Physics, Vrije Universiteit) at a probe temperature of 27 °C, using the WEFT pulse sequence for suppression of the residual HO^{2}H signal [19]. Spectra were recorded with a spectral width of 4000 Hz, collecting 64–256 free induction decays of 16 K complex data points. The resolution of the spectra was enhanced by Lorentzian-to-Gaussian transformation, and the final spectra were baseline corrected with a polynomal function. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) [20].

Determination of the stability of α3-GalT

Samples of 5 mU of purified α3-GalT were incubated at 4 °C and at 37 °C in 100 µl of a mixture having the same composition as the mixtures used for the syntheses. For comparison, samples of 5 mU α3-GalT were incubated at 4 °C and at 37 °C in 100 µl 30 mM Tris/maleate, pH 6.5. Aliquots of the mixtures, taken at various times of incubation, were assayed for α3-GalT activity.

Results

The following aspects of the enzymatic synthesis of the Galα1→3Galβ1→4GlcNAcβ1→O-R sequence were studied.

Time course of the synthesis of Galα1→3Galβ1→4GlcNAcβ1→O-R

The time course of the formation of Galβ1→4GlcNAcβ1→O-R and Galα1→3Galβ1→4GlcNAcβ1→O-R was established for two incubations on a 1 ml scale, with initial GlcNAcβ1→O-R concentrations of 2.5 and 5.0 mM, respectively. Aliquots of the incubation mixtures were analysed by HPEAC, after isolation of the GlcNAcβ1→O-R containing compounds using SepPak C_{18}. A typical elution pattern is shown in Fig. 2. Clearly, regardless of its

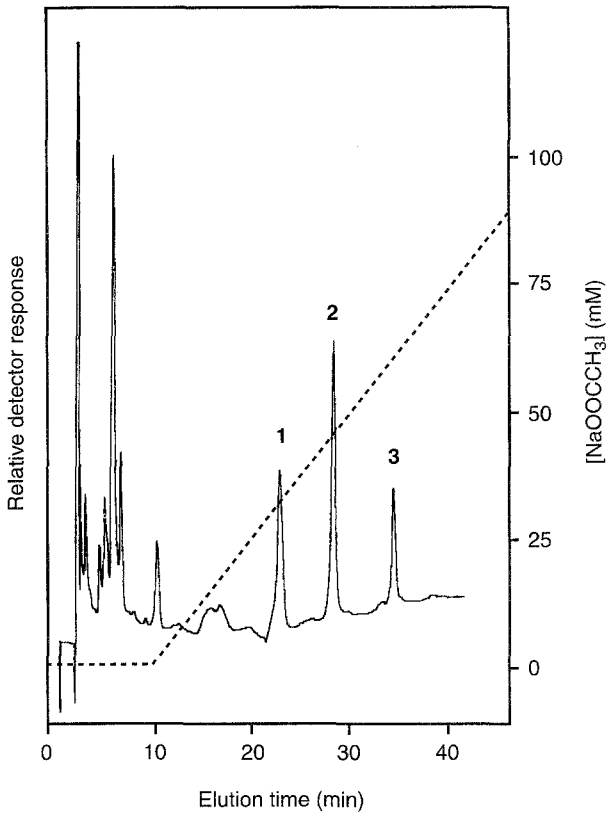

Figure 2. High-performance anion-exchange chromatography of the products formed after 2 h of the incubation of 2.5 mM GlcNAcβ1→O-R with α3-GalT and β4-GalT. The products were isolated using a SepPak C_{18} cartridge. 1, GlcNAcβ1→O-R; 2, Galβ1→4GlcNAcβ1→O-R; 3, Galα1→3Galβ1→4GlcNAcβ1→O-R. The CarboPac PA-1 column was eluted at a flow rate of 1 ml min^{-1} with a gradient of $NaOOCCH_3$ in 0.1 M NaOH as indicated.

starting concentration, GlcNAcβ1→O-R is quantitatively β4-galactosylated to yield Galβ1→4GlcNAcβ1→O-R as the intermediate product (see Fig. 3). The formation of Galα1→3Galβ1→4GlcNAcβ1→O-R follows that of the intermediate, but a complete conversion is not obtained. After 88 h of incubation, the yields relative to GlcNAcβ1→O-R of the disaccharide and the trisaccharide products are 18% (0.4 µmol) and 82% (2.1 µmol), respectively, in the case of a starting concentration of 2.5 mM GlcNAcβ1→O-R. The yields of these products at a starting concentration of 5.0 mM GlcNAcβ1→O-R are 56% (2.8 µmol) and 44% (2.2 µmol), respectively.

Product identification

Upon fractionation of the products of the 5 ml scale incubation on Bio-Gel P-4 (not shown), two carbohydrate containing fractions were obtained. The ^{1}H-NMR data (Table 1) of the product in the second fraction (14.5 µmol) are in accordance to those of Galβ1→4GlcNAcβ1→O-R [21]. The ^{1}H-NMR spectrum (Fig. 4; Table 1) of the

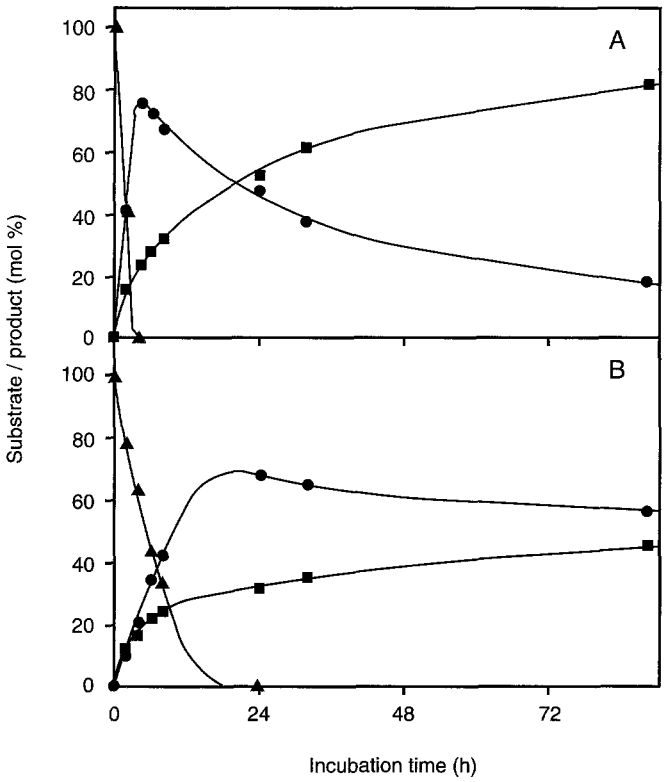

Figure 3. Time-course of the formation of Galβ1→4GlcNAcβ1→O-R (●) and Galα1→3Galβ1→4GlcNAcβ1→O-R (■), and the disappearance of GlcNAcβ1→O-R (▲). The substrate GlcNAcβ1→O-R was incubated with α3-GalT and β4-GalT at initial concentrations of 2.5 mM (A) and 5.0 mM (B), respectively.

product in the first fraction (10.5 μmol) shows the presence of Galα1→3Galβ1→4GlcNAcβ1→O-R. Compared to the spectrum of Galβ1→4GlcNAcβ1→O-R, additional signals stemming from the α-Gal residue are present at δ 5.145, (H-1; $J_{1,2}$ 3.8 Hz), δ 4.021 (H-4) and δ 4.192 (H-5). Furthermore, the H-1 and H-4 signals of the β-Gal residue have shifted downfield (Δδ 0.072 and Δδ 0.256, respectively) which is in agreement with the shifts observed for the α3-galactosylation of Galβ1→4GlcNAc [22].

Enzyme stability

The stability of sucrose synthase, UDP-Glc 4′-epimerase and β4-GalT under the incubation conditions has been investigated previously (Zervosen and Elling, unpublished results). These enzymes were found to be stable for at least 11 days. In order to investigate whether incomplete conversion of the intermediate product to the final trisaccharide Galα1→3Galβ1→4GlcNAcβ1→O-R could be due to inactivation of α3-GalT upon prolonged incubation, we investigated the stability of this enzyme. We found that α3-GalT remained fully active in the incubation mixture for at least 7 days, either when stored at 37 °C or at 4 °C. Remarkably, kept in 30 mM Tris/maleate, pH 6.5, α3-GalT was completely inactivated within 4 days, both when stored at 37 °C and at 4 °C.

Discussion

The trisaccharide Galα1→3Galβ1→4GlcNAcβ1→O-R is of particular interest as a ligand for the immunoadsorption or neutralization of human anti-α3-Gal antibodies which cause hyperacute rejection of xenotransplanted organs [2, 3]. In addition, oligosaccharides containing this terminal trisaccharide element will find an application as probes to investigate Galα1→3Gal mediated cell-cell interaction.

The chemical synthesis of Galα1→3Galβ1→4GlcNAcβ1→benzyl from synthetic precursors on a 50 μmol scale has been reported [23]. Such a synthesis involves multiple protection and deprotection steps, and purification of the intermediates. In this report, we present a simple and convenient method for the enzymatic synthesis of the Galα1→3Galβ1→4GlcNAc sequence. Only four enzymes, combined in a single incubation mixture, were used to carry out *in situ*

Table 1. ^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the products Galβ1→4GlcNAcβ1→O-R and Galα1→3Galβ1→4GlcNAcβ1→O-R. Chemical shifts are given at 27 °C and were measured in ^2H_2O relative to internal acetone (δ 2.225 [20]). Signals stemming from the spacer R are not presented in the Table

Reporter group	*Residue*	*Chemical shift in*		
		Substrate GlcNAcβ1→O-R (ppm)	*Intermediate product Galβ1→4GlcNAcβ1→O-R (ppm)*	*Final product Galα1→3Galβ1→4GlcNAcβ1→O-R (ppm)*
H-1	GlcNAc	4.499	4.521	4.525
	Galβ	–	4.472	4.544
	Galα	–	–	5.145
H-4	Galβ	–	3.925	4.181
	Galα	–	–	4.021
H-5	Galα	–	–	4.191
NAc	GlcNAc	2.032	2.030	2.031

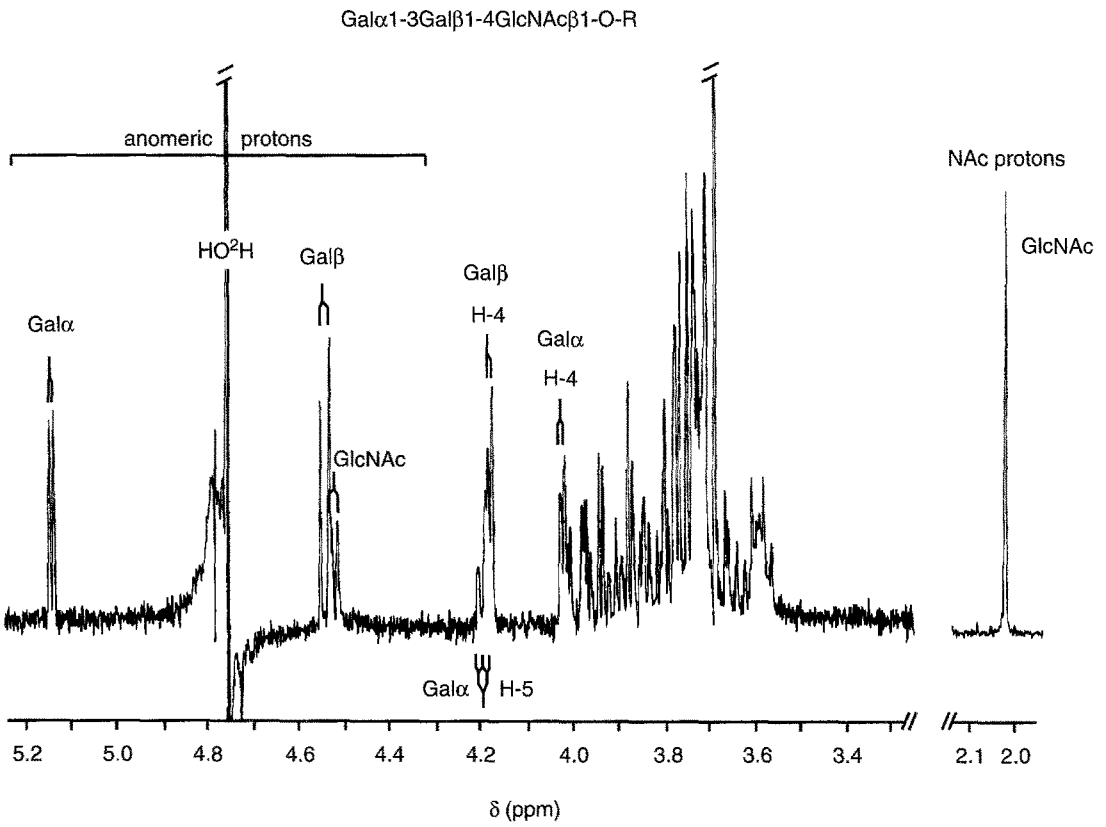

Figure 4. Structural-reporter-group regions of the resolution enhanced ^{1}H-NMR spectrum of the incubation product Galα1→3Galβ1→4GlcNAcβ1→O-R, recorded in ^2H_2O at 27 °C. The relative scale of the NAc protons region differs from that of the rest of the spectrum.

regeneration of UDP-Gal and the two subsequent galactosyl transfer steps to produce Galα1→3Galβ1→4GlcNAcβ1→O-R from GlcNAcβ1→O-R (Fig. 1).

The incubations we performed yielded approximately 2.2 μmol of Galα1→3Galβ1→4GlcNAcβ1→O-R per ml of reaction mixture. Higher yields per volume might be obtained by using a more concentrated α3-GalT preparation to convert a larger portion of the intermediate Galβ1→4GlcNAcβ1→O-R to the final product. The system lends itself for being scaled up easily to a scale of 50 μmol or more, because the amounts of enzymes needed are readily available. Sucrose synthase has been purified from rice on a large scale [15], β4-GalT and UDP-Glc 4′-epimerase are commercially available and the recombinant α3Gal-T is produced in our laboratory in large amounts in insect cell cultures.

A quantitative conversion of the intermediate Galβ1→4GlcNAcβ1→O-R to the final product Galα1→3Galβ1→4GlcNAcβ1→O-R was not obtained under the conditions employed. To find out if this is due to inactivation of the enzymes during prolonged incubation, their stability has been investigated. For β4-GalT, sucrose synthase and UDP-Glc 4′-epimerase, it has been shown that they remain active for at least 11 days in the specific incubation mixture we have used (Zervosen and Elling, unpublished results). The soluble recombinant bovine α3-GalT turned out to be stable for at least 7 days in this incubation mixture. We suggest that the presence of 0.5 M sucrose, necessary to drive the UDP-Gal regeneration cycle, has a stabilizing effect on the enzymes. Other possible reasons for the non-quantitative conversion of the intermediate to the final product are currently under investigation. Obviously, α3-GalT is not inhibited by UDP because this is reused for UDP-Gal generation. Also, we have found that α3-GalT is not inhibited by its product Galα1→3Galβ1→4GlcNAcβ1→O-R at a 5 mM concentration (Hokke and Van den Eijnden, unpublished results).

We have used GlcNAcβ1→O-$(CH_2)_8COOCH_3$ as an acceptor in the present experiments, since its hydrophobic tail allows fast and simple purification of the products from the incubation mixture, using SepPak C_{18} cartridges. In view of the acceptor specificity of α3-GalT and β4-GalT [9, 17, 24, 25], many other terminal-GlcNAc-containing compounds may serve as starting acceptors. Also, the flexibility of the transferases with respect to the aglycon at the reducing terminus will allow the use of various spacer molecules. The spacer may enable a

specific purification method like in the present experiments, but can also be employed for coupling of the oligosaccharides to carriers or other functional molecules like fluorescent labels, thus allowing the synthesis of multivalent ligands and versatile probes to study biological interactions.

In conclusion, this paper describes the efficient enzymatic synthesis of the Galα1→3Galβ1→4GlcNAcβ1→O-R sequence. Within our programme on the synthesis of potential ligands for cell-surface, carbohydrate-recognizing adhesion molecules, investigations are now aimed at the further exploration of this method for the production of various other types of galactosylated oligosaccharides.

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