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# Synthesis of Galβ1-3GlcNAc and Galβ1-3GlcNAcβ-SEt by an Enzymatic Method Comprising the Sequential Use of β-Galactosidases from Bovine Testes and *Escherichia coli*

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**Galβ1-3GlcNAc (1) and Galβ1-3GlcNAcβ-SEt (2) were synthesized on a 100 mg scale by the transgalactosylation reaction of bovine testes β-galactosidase with lactose as donor and *N*-acetylglucosamine and GlcNAcβ-SEt as acceptors. In both cases the product mixtures contained unwanted isomers and were treated with β-galactosidase from *Escherichia coli* which has a different specificity, under conditions favouring hydrolysis, yielding besides the desired products, monosaccharides and traces of trisaccharides. The products were purified to >95% by gel filtration, with a final yield of 12% of 1 and 17% of 2, based on added acceptor. In a separate experiment Galβ1-6GlcNAcβ-SEt (3) was synthesized by the transglycosylation reaction using β-galactosidase from *Escherichia coli*. No other isomers were detected. Compound 3 was purified by HPLC.**

With the increasing interest in biologically active carbohydrates has come an appreciation of the search for new methods of oligosaccharide synthesis, one of which utilizes glycosidases. Glycosidases can be used either by the reversal of the hydrolysis reaction [1, 2] or by the transglycosylation reaction [3, 4]. Because of the often limited aglycone specificity of these enzymes [5, 6], the product mixture can become complex [7, 8] and the purification of desired products cumbersome. In a previous study [9] we reported a new method which exploits the sequential use of β-galactosidases from bovine testes and *E. coli*. The latter enzyme with a slightly different specificity [10, 11], was used to hydrolyze unwanted structures formed by the bovine testes enzyme, thereby giving a mixture easily fractionated by gel chromatography.

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In this paper we apply the method to the synthesis of the two related structures **1** and **2**. The structure **1** is a constituent of the human milk oligosaccharides, e.g. lacto-*N*-tetraose, lacto-*N*-fucopentaose I and II, and also a component of the blood group determinants of the ABO-system [12]. The thioglycoside **2** can be used as a building block (after *O*-protection) in the synthesis of oligosaccharides [13] of which the structure, Galβ1-3GlcNAcβ is a part. Thus combining both chemical and enzymatic approaches it is possible to increase the efficiency and diversity of oligosaccharide synthesis.

## Materials and Methods

### *Materials*

*E. coli* β-galactosidase (E.C. 3.2.1.23) grade 6 (specific activity 80-150 U/mg protein, assayed as below), lactose, *N*-acetylglucosamine and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GlcNAcβ-SEt was synthesized as published [14]. Bovine testes β-galactosidase (E.C. 3.2.1.23; 0.14 U/mg protein) was a crude preparation obtained as previously described [9]. Acetonitrile was obtained from Labscan Ltd (Dublin, Ireland) and was of Far u.v. grade. All other chemicals were of analytical grade or better.

### *Enzyme Assays*

β-Galactosidase from bovine testes was incubated with 2 mM ONPG in 50 mM sodium phosphate-citrate buffer, pH 4.3 containing 0.02 % sodium azide. Samples were withdrawn at intervals and diluted eight times with 0.2 M $Na_2CO_3$, prior to spectrophotometric measurements at 420 nm. β-Galactosidase from *E. coli* was incubated with 2 mM ONPG in 50 mM sodium phosphate buffer, pH 7.0 with 1 mM $MgCl_2$ and 0.02 % sodium azide, and assayed directly at 420 nm. All assays were run at 37°C. One unit (U) was defined as the hydrolysis of 1 µmol ONPG/min under the above conditions.

### *HPLC Analyses*

Transgalactosylation and hydrolysis reactions were monitored by HPLC on an LKB (Bromma, Sweden) equipment with either a 2151, variable wavelength monitor set to 195 nm, or a refractive index (RI) detector from Tecator (Höganäs, Sweden). Separations were performed on a Merck (Darmstadt, FRG) Lichrosorb $NH_2$-column with 70% aqueous acetonitrile. HPLC purifications were performed on a semi-preparative Lichrosorb $NH_2$-column. The reactions were normally followed by $A_{195}$-detection with the sensitivity of the monitor set in such a way that only acetamido structures were seen. This was more sensitive than RI-detection, but when detection of contaminating di- and trisaccharides was necessary, RI-detection was used. RI-detection was also used in the purifications by HPLC.

### *Transgalactosylation*

Reactions were performed in 50 mM sodium phosphate-citrate buffer, pH 4.3 with 0.02% sodium azide, at 37°C, with 20% (w/w) lactose and 5% of the corresponding acceptor.

Bovine testes β-galactosidase (1.8 U/g of added lactose) was used. Maximum yields occurred after 20-30 h. The reactions were terminated by heating to 90°C for 10 min.

*Hydrolysis*

The reaction mixtures from the transgalactosylations were diluted 10 times with 50 mM sodium-phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$ and 0.02% sodium azide. The pH was checked and if necessary, adjusted to 7.0. *E. coli* β-galactosidase was added (1470 U/g lactose), and the reactions were run at 37°C for 22 h or more. The hydrolyses were stopped by heating to 90°C for 10 min. When *N*-acetylglucosamine was used as acceptor, it was necessary to lower the pH to approximately 4.3 before heating, in order to avoid breakdown of **1**.

*Isolation*

The lyophilized mixtures from reactions based on 2 g lactose and 0.5 g acceptor were dissolved in water with 2.7 mM β,β,β-trichlorobutyl alcohol and filtrated on a Millex-HV filter, Waters (Milford, U.S.A.). The solution was then applied on a Bio-Rad (Richmond, CA, U.S.A.) Bio-Gel P-2 column (5 x 95 cm) and eluted with the same solvent to separate most of the monosaccharides from the desired products. The effluent was monitored spectrophotometrically at 206 nm and by refractive index detection. U.v.-positive fractions (indicating the presence of acetamido sugars) were pooled and analyzed by HPLC prior to evaporation to dryness. Fractions containing the desired disaccharides were again dissolved in the water solution, applied to the same P-2 column and eluted as above. The effluent was recirculated on the column until baseline separation of the desired products was achieved. The fractions containing **1** and **2** were pooled and lyophilized.

*Synthesis of Galβ1-6GlcNAcβ-SEt (**3**)*

The structure **3** was synthesized under conditions identical to the transglycosylation reaction yielding **2**, except that the *E. coli* enzyme was used instead of the bovine testes enzyme and the reaction performed in the corresponding buffer. The hydrolysis step was also left out. According to GLC-MS, the yield was 28%, and no other isomers of Galβ-GlcNAcβ-SEt were detected. The purification of **3** was done with HPLC, leading to a purity of >95% according to NMR.

*Analytical Procedure*

Methylation of the product mixture using GlcNAcβ-SEt as acceptor was done by solid NaOH/DMSO/MeI according to the method of Gunnarsson [15]. Methylation of the product mixture using *N*-acetylglucosamine as acceptor was done either by the Hakomori procedure [16] or by solid NaOH/DMSO/MeI after reduction. Methylation analysis was performed as previously described [15, 17]. $^1$H-NMR and $^{13}$C-NMR spectra were obtained from solutions of the compounds in $^2H_2O$ (internal acetone,2.225 ppm) at 27°C with a Bruker 500 MHz instrument. GLC was carried out on a Perkin-Elmer Sigma 1 gas chromatograph equipped with a flame ionization detector. Separations were performed on a DB-1, fused silica capillary column (30 m x 0.25 mm) at (a) 160-330°C for methylated oligosaccharides and

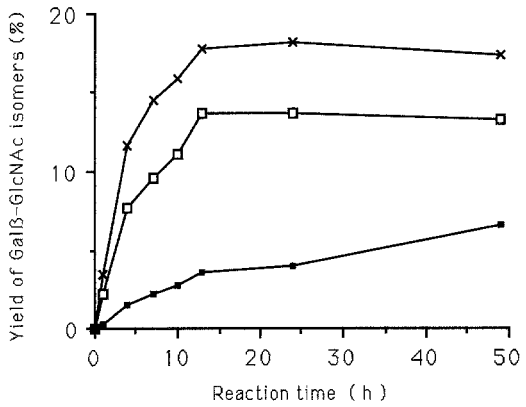


**Figure 1.** Results from a transgalactosylation with *N*-acetylglucosamine as acceptor. Yields are expressed as % (w/w) of added *N*-acetylglucosamine and are calculated from GLC chromatograms.

x, Galβ1-3GlcNAc; □, Galβ1-4GlcNAc; ■, Galβ1-6GlcNAc.

methylated oligosaccharide alditols and (b) 160-250°C for partially methylated alditol acetates.

GLC-MS was performed on a VG Masslab 1250 quadrupole instrument linked to a Hewlett-Packard 5790 gas chromatograph equipped with a split-splitless injector and the appropriate column. The spectra were recorded at 70 eV with an ion-source temperature of 200°C and processed by an PDP 11/23 on-line computer system.

## Results and Discussion

### *Galβ1-3GlcNAc (**1**)*

Transgalactosylations with *N*-acetylglucosamine as acceptor were followed by HPLC and also by GLC and GLC-MS after reduction and permethylation of the material. GLC-MS showed three isomers containing Hex-HexNAc-ol, identified by their mass spectra by the presence of m/z 219, 187, 155 (aA-series of the non-reducing end) and m/z 276 ($J_2$-fragment from the reducing end of HexNAc-ol). Cleavages in the alditol chain also indicated the type of linkages by the presence of m/z 130, 174 and 218 for a (1-6)-linkage, the absence of m/z 218 for a (1-4)-linkage and the absence of m/z 218 and 174 for a (1-3)-linkage [18, 19]. The (1-3)-isomer had the shortest retention time on the column, followed by the (1-4)- and the (1-6)-isomer, in that order. The type of linkages was further confirmed by methylation analysis, which showed the presence of non-reducing, terminal galactose and 3-,4- and 6-substituted *N*-acetylglucosamine. The rate of formation of the isomers is shown in Fig. 1. The yields were determined as % (w/w) of added *N*-acetylglucosamine. As seen in the Fig. 1, **1** was formed at the greatest rate and reached its maximum yield after 20-30 h. Galβ1-6GlcNAc was formed more slowly than the others and had not reached its maximum after 49 h.

HPLC analysis could not resolve the different isomers and therefore the formation of **1** *per se* could not be followed. Instead, the transgalactosylations were terminated well after the total yield of the different isomers had levelled off at approximately 15 h. Because of the very small differences in the yield of **1** between 15 and 50 h, this method was sufficient.

In the hydrolysis reaction following the transgalactosylation reaction, lactose and contaminating isomers were rapidly hydrolyzed, whereas no hydrolysis of **1** was observed after 22 h, apart from a possible slight initial hydrolysis. After 1 h the (1-6)-isomer was hydrolyzed, whereas 6 h were needed to remove the (1-4)-isomer. After 22 h all contaminating disaccharides and all but traces of the trisaccharides were hydrolyzed (data not shown).

Purification of **1** from a synthesis based on 2 g lactose and 0.5 g *N*-acetylglucosamine, by gel chromatography gave a fraction consisting of 62 mg. GLC-MS analysis of the reduced and permethylated fraction showed one compound consisting of Hex-HexNAc-ol, which after methylation analysis was identified as Gal1-3GlcNAc. $^{1}$H- and $^{13}$C-NMR confirmed this and that the galactose residue was in the β-configuration [20]. The purity of **1** was over 95% and the yield 12%, calculated on the amount of added *N*-acetylglucosamine. $^{1}$H-NMR ($^{2}H_2O$): δ 5.18/4.48 (α/β, d $J_{1,2}$ 3.4/7.9 Hz, H-1); 4.46/4.24 (d $J_{1,2}$ 7.7/7.7 Hz, H-1', due to α,β-anomerization of the *N*-acetylglucosamine); 2.03 (s, C**H**$_3$CON-). $^{13}$C-NMR ($^{2}H_2O$): δ 91.9/95.6 (α/β, C-1); 104.3/104.4 (C-1', due to α,β-anomerization of the *N*-acetylglucosamine); 22.9/23.1 (**C**H$_3$CON-, due to α,β-anomerization of the *N*-acetylglucosamine); 175.4 (CH$_3$**C**ON-). $[\alpha]_D$ = +14.0°.

*Galβ1-3GlcNAcβ-SEt (**2**)*

In transgalactosylations using GlcNAcβ-SEt as acceptor, three isomers of Hex-HexNAc-SEt were formed, according to GLC-MS after methylation of the product mixture. The mass spectra of the isomers showed the presence of m/z 290, which is consistent with the $bA_1$-fragment of a HexNAc-SEt, and the aA-series m/z 219, 187, 155 for a non-reducing terminal Hex [18, 19]. The type of linkage could not be established by the mass spectra and therefore the identification of the different GLC-MS peaks was undertaken after partial HPLC purification of the reaction mixture. HPLC analysis showed two fully separated u.v.-absorbing peaks, besides free GlcNAc-SEt. Peak 1 contained 3- and 4-substituted GlcNAc-SEt, besides non-reducing galactose, according to methylation analysis. On GLC-MS of permethylated material from peak 1, two isomers of Hex-HexNAc-SEt were found. The discrimination between the (1-3)-and the (1-4)-isomer was done after hydrolysis of the mixture by *E. coli* β-galactosidase, which left one isomer intact. This was shown to be Gal1-3GlcNAc-SEt according to GLC-MS and methylation analysis. The second HPLC peak contained one isomer, according to GLC-MS of permethylated material, and on methylation analysis it was identified as Gal1-6GlcNAc-SEt by the presence of non-reducing terminal galactose and 6-substituted GlcNAc-SEt. The isomers were eluted in the same order (1-3, 1-4 and 1-6) on GLC-MS, as for the Gal-GlcNAc isomers. The transgalactosylation reaction could not be quantified by GLC due to the low response with flame ionization detection. Instead the reaction was followed by GLC-MS and quantified against an internal standard (perseitol) after determining the response factors of the Gal-GlcNAc-SEt isomers. $^{1}$H-and $^{13}$C-NMR of the purified Gal1-3GlcNAc-SEt confirmed this structure and showed the β-configuration of galactose [20].

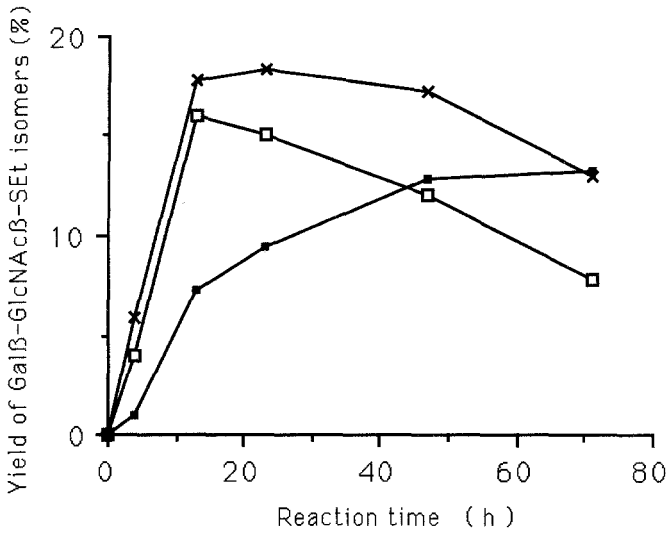


**Figure 2.** Results from a transgalactosylation using GlcNAcβ-SEt as acceptor. Yields are calculated from GLC-MS chromatograms and expressed as % (w/w) of added GlcNAcβ-SEt.
x, Galβ1-3GlcNAcβ-SEt; □, Galβ1-4GlcNAcβ-SEt; ■, Galβ1-6GlcNAcβ-SEt.

The rate of formation of the isomers is illustrated in Fig. 2. Compound **2** and Galβ1-4-GlcNAcβ-SEt were formed initially at the same rate but the (1-4)-isomer was hydrolyzed slightly more rapidly. The (1-6)-isomer was formed most slowly of the isomers in a way analogous to Galβ1-6GlcNAc. Because of the approximately equal rate of formation of **2** and its (1-4)-isomer, and because the (1-6)-isomer appears as a separate peak, HPLC analysis could be used efficiently to monitor the transgalactosylation reaction.

During the hydrolysis of the transgalactosylation reaction the (1-6)-isomer was hydrolyzed after less than 30 min and the (1-4)-isomer after 2 h, whereas disaccharides and most of the trisaccharides were not removed until 22 h. No hydrolysis of **2** was observed under these conditions (data not shown).

After purification and isolation of **2** by gel chromatography, 84 mg were obtained from a synthesis based on 500 mg GlcNAcβ-SEt. By $^1$H-NMR it was seen that the purity was well over 95%. Methylation analysis, $^{13}$C-NMR and $^1$H-NMR confirmed the structure. The overall yield was 17% calculated on added GlcNAcβ-SEt. $^1$H-NMR ($^2H_2O$): δ 4.67 (d, $J_{1,2}$ 9.8 Hz, H-1); 4.45 (d, $J_{1,2}$ 7.9 Hz, H-1'); 2.02 (s, C**H**$_3$CON-); 1.25 (t, C**H**$_3$CH$_2$S-); 2.77,2.70 (m,m, CH$_3$C**H**$_2$S-). $^{13}$C-NMR ($^2H_2O$): δ 84.6 (C-1); 104.2 (C-1'); 54.3, 84.3, 69.4, 80.2, 61.6 (C-2,3,4,5,6); 71.4, 73.2, 69.3, 76.0, 61.8 (C-2',3',4',5',6'); 23.0 (**C**H$_3$CON-); 175.2 (CH$_3$**C**O-N-); 15.0 (**C**H$_3$CH$_2$S-); 25.1 (CH$_3$**C**H$_2$S-). $[\alpha]_D$ = -18.0°.

*Transgalactosylation*

The reactions were performed with a crude preparation of the bovine testes enzyme, which is important when considering large scale synthesis. Problems often occurring with crude preparations due to contaminant enzyme activities such as proteases or other glycosidases were not observed under the reaction conditions above. Significantly, the crude enzyme preparation contained large amounts of β-*N*-acetylglucosaminidase activity, but this contaminating activity had no effect under the reaction conditions used.

Although glycosidases have a limited aglycone specificity, changing the substituents of the aglycone often affects the results of both the transglycosylation and the hydrolysis reaction [21-23]. In a previous study [9] based on the same method as applied here, we reported the synthesis of Galβ1-3GalNAc. In that study we did not detect any Galβ1-4GalNAc, but as seen in this work, changing the acceptor from *N*-acetylgalactosamine to *N*-acetylglucosamine resulted in large amounts of the (1-4)-isomer, and also a significantly lower yield of the (1-3)-isomer. In view of this it was therefore interesting to notice the rather limited effects that the β-SEt group had on the enzymes acceptor selectivity in the different reactions. In future studies we intend to check the effects of the aglycone structure, by preferentially using acceptors larger than GlcNAcβ-SEt.

*Hydrolysis*

The hydrolysis reactions were performed with substantial amounts of enzyme. This was done because several contaminating structures were poor substrates and some monosaccharides have an inhibitory effect on the *E. coli* β-galactosidase [24].

The results of the hydrolysis reactions were at first somewhat surprising. In contrast to our results, Li and co-workers [25, 26] showed that Galβ1-3GlcNAc was hydrolyzed more rapidly than its (1-4)-isomer by the β-galactosidase from *E. coli*. Although the experiments by Li *et al.* were performed with single substrate incubations and ours were complicated mixtures and therefore not quite comparable, the results were not expected. However, the results could be satisfactorily explained if the $K_M$ value for Galβ1-3GlcNAc is significantly higher than for Galβ1-4GlcNAc.

## Concluding Remarks

Transglycosylations with glycosidases normally result in several isomers, although in some cases only one isomer is formed, as seen for instance in the synthesis of **3**. However, with the sequential use of glycosidases with partly overlapping specificities the problem with the formation of several isomers can be eliminated.

A crucial aspect of the method is to find matching enzyme pairs or a matching set of enzymes. This is presently not a trivial task, since only few glycosidases are sufficiently characterized with respect to their isomer specificities. Data available on hydrolysis can, however, in some cases be useful in predicting results also in transglycosylations. We are presently engaged in expanding the method to other enzymes and oligosaccharides.

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**References**

1 Abe J-I, Hizukuri S, Koizumi K, Kubota Y, Utamura T (1988) Carbohydr Res 176:87-95.
2 Johansson E, Hedbys L, Mosbach K, Larsson P-O, Gunnarsson A, Svensson S, Enzyme Microb Technol, in press.
3 Straathof AJJ, Vrijenhoef JP, Sprangers EPAT, van Bekkum H, Kieboom APG (1988) J Carbohydr Chem 7:223-38.
4 Usui T, Hayashi Y, Nanjo F, Ishido Y (1988) Biochim Biophys Acta 953:179-84.
5 Kobata A (1979) Anal Biochem 100:1-14.
6 Dey PM, Pridham JB (1972) Adv Enzymol 36:91-129.
7 Mozaffar Z, Nakanishi K, Matsumo R (1986) Appl Microb Biotechnol 25:224-28.
8 Toba T, Yokata A, Adachi S (1985) Food Chem 16:147-62.
9 Hedbys L, Johansson E, Mosbach K, Larsson P-O, Gunnarsson A, Svensson S, (1989) Carbohydr Res 186:217-23.
10 Hedbys L, Larsson P-O, Mosbach K, Svensson S (1984) Biochem Biophys Res Commun 123:8-15.
11 Distler JJ, Jourdian GW (1973) J Biol Chem 248:6772-80.
12 Kennedy JF, White CA (1983) Bioactive Carbohydrates in Chemistry, Biochemistry and Biology, Ellis Horwood Ltd, Chichester, England.
13 Fügedi P, Garegg PJ, Lönn H, Norberg T (1987) Glycoconjugate J 4:97-108.
14 Hough L, Taka MI (1956) J Chem Soc 2042-48.
15 Gunnarsson A (1987) Glycoconjugate J 4:239-45.
16 Hakomori S-I (1964) J Biochem (Tokyo) 55:205-8.
17 Björndal H, Hellerqvist CG, Lindberg B, Svensson S (1970) Angew Chem Int Edn Engl 9:610-19.
18 Kochetkov NK, Chizhov OS (1966) Adv Carbohydr Chem Biochem 21:39-93.
19 Lönngren J, Svensson S (1974) Adv Carbohydr Chem Biochem 29:41-106.
20 Bradbury JH, Jenkins GA (1984) Carbohydr Res 126:125-56.
21 Nilsson KGI (1987) Carbohydr Res 167:95-103.
22 Corfield AP, Wember M, Schauer R, Rott R (1982) Eur J Biochem 124:521-25.
23 van den Eijnden DH, Blanken WM, van Vliet A (1986) Carbohydr Res 151:329-35.
24 Deschavanne PJ, Viratelle OM, Yon JM (1978) J Biol Chem 253:833-37.
25 Li Y-T, Wan CC, Chien J-L, Li S-C (1978) Adv Exp Med Biol101:537-544.
26 Li S-C, Mazzotta MY, Chien S-F, Li Y-T (1975) J Biol Chem 250:6786-6791.