Pergamon

Tetrahedron Letters, Vol. 38, No. 1, pp. 133-136, 1997
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0040-4039/97 $17.00 + 0.00

PII: S0040-4039(96)02235-6

Glycosidase-Catalysed Synthesis of Di- and Trisaccharide Derivatives Related to Antigens Involved in the Hyperacute Rejection of Xenotransplants

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Abstract: A convenient enzymatic procedure, suitable for large scale preparation of the β-thioethyl 2-N-Teoc-derivative of Galα1-3Galβ1-4GlcNAc, is described (Teoc = 2,2,2-trichloroethoxycarbonyl). β-D-Galactosidase from *Bullera singularis* was used for the specific synthesis of Galβ1-4GlcNTeocβ-SEt from lactose and D-GlcNTeocβ-SEt. α-D-Galactosidase from coffee beans was used for the stereospecific and highly regioselective preparation of Galα1-3Galβ1-4GlcNTeocβ-SEt, employing D-Galα-OPhNO_2-p as glycosyl donor and Galβ1-4GlcNTeocβ-SEt as acceptor.
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It is known that ca 1 % of circulating human IgG binds to carbohydrate antigens which contain the Galα1-3Gal-determinant[1]. This determinant is found in small amounts on certain tumours[2] and in porcine tissues[3]. Due to the severe shortage of human donor organs for transplantation, there is an intense activity to avoid the antibody-mediated hyperacute rejection response in cross-species transplant rejection from pigs to man (xenotransplantation)[3]. Affinity columns, which contain immobilised saccharides with the Galα1-3Gal-terminus, have been shown to specifically remove human antibodies involved in the hyperacute rejection response[3,4]. Moreover, it has been shown that soluble Galα1-3Gal, the trisaccharide Galα1-3Galβ1-4GlcNAc and higher saccharides which contain this trisaccharide structure, inhibit the binding of human antibodies to pig cells[3,4], thus indicating the potential clinical use of such compounds or analogues thereof as inhibitors of the hyperacute rejection process.

The clinical application of carbohydrates which contain the Galα1-3Gal epitope depends on the availability of large quantities of the basic di-, tri- and higher oligosaccharide structures. The chemical synthesis of carbohydrates have been developed extensively the last decade[5]. However, the available chemical methods are not completely stereospecific and multi-reaction schemes are used, which complicates scaled-up production. Thus, there is a need for alternative methods.

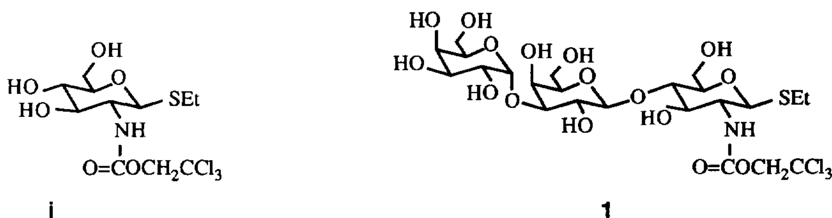
Glycosidases are stereospecific catalysts, which are readily available in large quantities and which allow the use of non-complicated sugars, such as simple saccharides or glycosides, as glycosyl donors[6]. The regioselectivity can in several cases be manipulated to achieve highly specific synthesis of the desired linkages[6-8], and glycosidase-catalysed reactions have been used for the preparation of a broad range of biologically active carbohydrate sequences[6-8], partially modified carbohydrates[8-10] and glycosides[8-12].

We have previously reported the convenient synthesis of different glycosides of the Galα1-3Gal epitope with molar yields of up to 65 %, employing α-galactosidase as catalyst and raffinose or D-Galα-OPhNO_2-p as glycosyl donor[7,12]. Moreover, the syntheses of partially modified Galα1-3Gal glycosides have been demonstrated[8,9]. Both enzyme and substrates are readily available in larger amounts, which facilitates bulk scale preparation of the Galα1-3Gal disaccharide, as well as glycosides and analogues thereof.

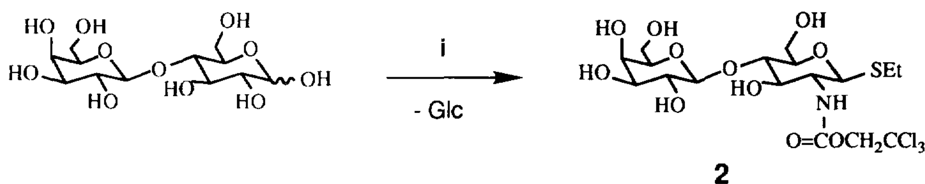
The versatility of thioethyl glycosides and of the 2-N-Teoc-protection group (Teoc = 2',2',2'-trichloroethoxycarbonyl) in carbohydrate syntheses have been well documented[5,13]. We were interested to

investigate if the 2-N-Teoc-protected D-glucosamine derivative GlcNTeocβ-SEt, **i**, can be used as acceptor in glycosidase-catalysed synthesis, since, if successful, this would facilitate large-scale synthesis of di- and trisaccharide intermediates, useful e.g in the preparation of xeno-antigens, glycosides and analogues.

This paper describes the synthesis of the trisaccharide derivative Galα1-3Galβ1-4GlcNTeocβ-SEt, **1**, employing two sequential glycosidase-catalysed reactions and **i** as the initial acceptor substrate.



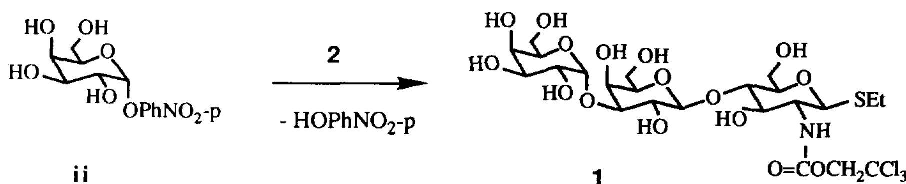
In the first glycosidase-catalysed reaction, the β-D-galactosidase from the yeast *Bullera singularis* was used for the direct conversion of lactose to the D-lactosamine derivative **2**, employing **i** as acceptor (Scheme 1). We have previously reported that the β-D-galactosidase from the yeast *Bullera singularis* catalyses the specific synthesis of different D-lactosamine (Galβ1-4GlcN) derivatives[10]. This enzyme tolerates modifications of the 2-amino group of glucosamine remarkably well. Thus, the 2-N-phthaloyl-derivative of lactosamine was prepared in multi-gramme quantities from lactose employing the 2-N-phthaloyl derivative of D-glucosamine as acceptor[10]. As shown here, the enzyme also tolerates the 2-N-Teoc protection group without decrease in specificity, and the above reaction gave the 2-N-Teoc protected lactosamine derivative **2** in a stereo- and regiospecific manner[14]. Thus, no other regioisomers were apparently produced in this reaction. Compound **2** was isolated to homogeneity by one column chromatographic step (Sephadex^R G10; water as eluent), which also quantitatively recovered the non-reacted acceptor in pure form. As reported before for other hydrophobic glycosides, e.g. nitrophenyl disaccharide glycosides[7,12], **i** and **2** were slightly retained on the Sephadex material compared to non-modified disaccharides, which facilitated separation and isolation. The procedure gave **2** in ca 20 % molar yield as calculated on added acceptor. The total yield of **2** from **i** can be increased by repeated synthesis with the recovered acceptor.



Scheme 1. β-D-Galactosidase-catalysed synthesis of Galβ1-4GlcNTeocβ-SEt.

In the second glycosidase-catalysed reaction, the α-D-galactosidase from green coffee beans was used for α1-3-galactosylation of the lactosamine derivative **2**, employing D-Galα-OPhNO_2-p, **ii**, as glycosyl donor (Scheme 2)[15]. The 2-N-Teoc derivative **1** was obtained in a stereospecific manner and with high regioselectivity (ca 85 % from HPLC of the reaction mixture; the α1-6-linked trisaccharide regioisomer was also formed). Isolation of **1** was achieved by extraction of the reaction mixture (removal of p-nitrophenol) followed by column chromatography (Sephadex^R G15; water as eluent), which also removed the regioisomer of compound **1** and recovered the non-reacted acceptor. Different separation materials may be used (e.g. reversed phase-silica, charcoal-Celite), but we found Sephadex to be advantageous, it was repeatedly used with water as eluent without decrease of separation efficiency. The procedure gave **1** in 9 % molar yield when

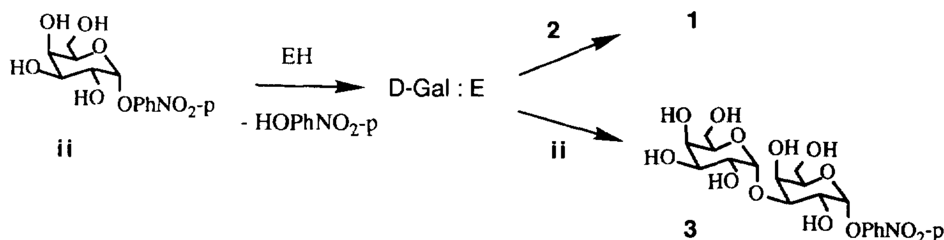
calculated on added acceptor and in ca 20 % yield when calculated on consumed donor. The total yield of **1** from **2** can be increased by repeated synthesis with the recovered acceptor.



Scheme 2. α-D-Galactosidase-catalysed synthesis of Galα1-3Galβ1-4GlcNTeocβ-SEt.

Glycosidase-catalysed transglycosylation reactions are believed to proceed via formation of a glycosyl-enzyme intermediate upon release of the donor aglycon[6,12,16]. The glycosyl-enzyme intermediate reacts with water or with various hydroxyl-group containing acceptors, which lead to hydrolysis or product formation, respectively. Thus, in addition to products **1** and **2**, galactose was formed in the above reactions. Moreover, the donor may act as an acceptor reacting with the galactosyl-enzyme intermediate. This caused the formation of trisaccharides in the β-D-galactosidase reaction, and of Galα1-3Galα-$OPhNO_2$-p, **3**, in the α-galactosidase-catalysed reaction (Scheme 3). The α-galactosidase-catalysed synthesis of **3** has been reported previously[7]. The above reactions did not complicate the isolation of the desired products, due to the different retention of products and side-products on the separation material.

In line with the above discussion, the α-galactosidase-catalysed reaction can be used for simultaneous preparative synthesis of both the p-nitrophenyl digalactoside **3** and the trisaccharide derivative **1**, if the reaction conditions are properly chosen (Scheme 3). Thus, when a high concentration of donor was used (1 M initial concentration of **ii**) at higher temperature (45 °C), **3** was formed in ca 250 mM concentration (ca 60 % molar yield as calculated on reacted donor) in addition to the formation of **1** in 55 mM concentration (14 % yield). The two products were separated by Sephadex chromatography. Compound **3** is useful, e.g. for the preparation of affinity ligands and the Galα1-3Gal disaccharide epitope[17].



Scheme 3. Simultaneous synthesis of **1** and **3** catalysed by α-D-galactosidase (EH).

Transglycosylation reactions are influenced by several parameters[6,12], and the above yields may be considerably improved after optimization. For example, by increasing the concentration of the acceptor **2** from 0.1 to 0.5 M, the molar yield of **1** in the α-galactosidase reaction was increased from ca 3 % to 12 %. Moreover, the formation of the disaccharide **3** in the α-galactosidase-catalysed reaction can be minimized, if desired, by keeping the concentration of glycosyl donor at a low level during the reaction (gradual addition of the donor). Work is now in progress for optimisation of the above reactions.

In conclusion, the trisaccharide derivative **1** was conveniently obtained in two sequential glycosidase-catalysed reactions employing the 2-N-Teoc-protected D-glucosamine derivative **i** as the initial acceptor. The here described procedure gives quick access to **1** in large quantities, since enzymes and substrates are readily available, a minimum of reaction steps are required and the productivity per reaction volume is relatively high. Compound **1**, as well as the disaccharide derivatives **2** and **3**, are useful intermediates for production of oligosaccharides and glycosides, evaluated e.g. as inhibitors of the hyperacute rejection of xenotransplants.

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14. In a typical reaction, lactose (10 g) and GlcNTeocβ-SEt (**i**; 5 g) were dissolved in 50 mM sodium acetate, (200 ml; pH 6.0) and crude β-galactosidase[11] (*Bullera singularis;* 1 g)was added. The reaction proceeded at 30 °C for three days. Column chromatography (Sephadex^R G10; 2 l; water as eluent), gave unreacted acceptor (**i**; 3.8 g) and product (**2**; 1.3 g). 400 MHz ^{1}H-NMR (selected data; D_2O-MeOD; 1:1); δ 4.62 (d, 10.6 Hz, H-1), 4.45 (d, 7.6 Hz, H-1'), 1.25 (t, 7.4 Hz, $-CH_3$), 2.73 (m, $-\underline{CH_2}CH_3$). ^{13}C NMR-data (D_2O-MeOD; 1:1): δ 85.28 (C-1), 57.39 (C-2), 74.80 (C-3), 79.67 (C-4), 79.87 (C-5), 61.34 (C-6), 104.07 (C-1'), 72.08 (C-2'), 73.78 (C-3'), 69.67 (C-4'), 76.45 (C-5'), 62.06 (C-6'); Teoc-group: 157.03 (C=O), 75.26 (CH_2), 96.31 (CCl_3).
15. In a typical reaction, Galα-OPhNO$_2$-p (150 mg) and Galβ1-4GlcNTeocβ-SEt (**2**; 1.05 g) were dissolved in 50 mM sodium phosphate (4 ml, pH 6.5) and α-galactosidase (coffee beans; 10 mg) was added. The reaction proceeded at room temperature with gradual addition of donor until 300 mM p-nitrophenol had been formed (60 h). The reaction mixture was extracted with methylene chloride, followed by column chromatography (Sephadex G10; 0.4 l; water as eluent), which gave product (**1**; 120 mg). 400 MHz ^{1}H-NMR (selected data; D_2O); δ 4.68 (d, 10.4 Hz, H-1), 4.56 (d, 7.6 Hz, H-1'), 5.15 (d, 3.6 Hz, H-1''), 1.25 (t, 7.3 Hz, $-CH_3$), 2.74 (m, $-\underline{CH_2}CH_3$). ^{13}C NMR-data (D_2O): δ 85.09 (C-1), 57.25 (C-2), 74.77 (C-3), 78.15 (C-4), 79.64 (C-5), 61.22 (C-6), 103.7 (C-1'), 70.51 (C-2'), 75.98 (C-3'), 65.75 (C-4'), 79.49 (C-5'), 61.82 (C-6'), 96.35 (C-1''), 69.10 (C-2''), 70.20 (C-3''), 70.03 (C-4''), 71.75 (C-5''), 61.91 (C-6''); Teoc-group: 157.5 (C=O), 75.2 (CH_2), 95.81 (CCl_3).
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(*Received in UK* 11 *October* 1996; *revised* 8 *November* 1996; *accepted* 15 *November* 1996)