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A SIMPLE STRATEGY FOR CHANGING THE REGIOSELECTIVITY OF GLYCOSIDASE-CATALYSED FORMATION OF DISACCHARIDES: PART II, ENZYMIC SYNTHESIS *in situ* OF VARIOUS ACCEPTOR GLYCOSIDES*†

KURT G. I. NILSSON
Swedish Sugar Co. Ltd., R & D, Box 6, S-232 00 Arlöv (Sweden)
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ABSTRACT

β-D-Galactosidase induced the formation of allyl, benzyl, and trimethylsilylethyl β-D-galactopyranosides on a 1–20-g scale from lactose and allyl alcohol, benzyl alcohol, and trimethylsilylethanol, respectively. Similarly, α-D-galactosidase catalysed the formation of allyl α-D-galactopyranoside from raffinose and allyl alcohol. The galactosides were used as acceptors for the preparation of the following disaccharide glycosides: β-D-Gal-(1→3)-β-D-Gal-$OCH_2CH{=}CH_2$, β-D-Gal-(1→6)-β-D-Gal-$OCH_2CH{=}CH_2$, β-D-Gal-(1→3)-β-D-Gal-OBn, β-D-Gal-(1→6)-β-D-Gal-OBn, β-D-Gal-(1→3)-β-D-Gal-$OCH_2CH_2SiMe_3$, and α-D-Gal-(1→3)-α-D-Gal-$OCH_2CH{=}CH_2$. The β-D-galactosidase-catalysed reactions were efficient enough to allow the one-pot preparation of the various β-linked mono- and digalactosides from lactose and alcohol.

INTRODUCTION

Glycoconjugates are involved in several processes in living organisms[1,2]. The chemical synthesis of such structures is often cumbersome[3], and the development of biosynthesis strategies is attractive because of the high efficiency, substrate specificity, and stereospecificity and regioselectivity of enzymes[4].

Although glycosidases have long been known to catalyse the stereospecific formation of glycosidic bonds by reversed hydrolysis or transglycosidation[5], the preponderant formation of (1→6) linkages and the difficulties in the isolation of products have hampered their wider use for synthesis of biologically active carbohydrates[6]. However, the regioselectivity of glycosidase-catalysed formation of oligosaccharides can be manipulated and the isolation of products simplified by using the appropriate glycosides as acceptors[7]. Thus, it was possible to manipulate an α-D-galactosidase to catalyse the preponderant formation of α-(1→2)-, α-(1→3)-, or α-(1→6)-linked digalactosides by using α-D-Gal-$OPhNO_2$-*o*, α-D-Gal-OMe, or

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β-D-Gal-OMe as acceptor, respectively. Methyl and nitrophenyl glycosides of various disaccharides were prepared in yields of 10–40% depending on the conditions[7,8].

This approach has now been extended to other, cheaper donors (raffinose, lactose) and to acceptor allyl, benzyl, and trimethylsilylethyl glycosides which are suitable for temporary anomeric protection, polymerisation, preparation of affinity labels, spacer arms, *etc.* The acceptor glycosides were prepared enzymically from lactose or raffinose and the respective alcohol. The enzyme-catalysed one-pot preparation of the β-linked mono- and di-galactosides from lactose and alcohol is also described. A simplified scheme[9,10] for these β-D-galactosidase-catalysed reactions is given below, where A symbolises the alcohol and E the enzyme.

$$\begin{array}{ccccc} & & \text{Gal} + \text{E} & & \\ & & \Downarrow\Uparrow\ \text{H}_2\text{O} & & \\ & -\text{Glc} & & \text{A} & \\ \text{Lactose} + \text{E} & \rightleftarrows & \text{E} \cdot \text{Gal} & \rightleftarrows & \text{Gal–A} + \text{E} \\ & & \Uparrow\Downarrow\ \text{Gal–A} & & \\ & \text{Gal–Gal–A} + \text{E} & & & \end{array}$$

EXPERIMENTAL

General. — α-D-Galactosidase (EC 3.2.1.22; coffee bean), β-D-galactosidase (EC 3.2.1.23; *E. coli*, grade VIII), and jack-bean meal were obtained from Sigma.

The general methods for isolation and characterisation of the products were as reported[7,11,12]. Thus, solvents were removed with a rotary evaporator and then at <0.1 Torr. T.l.c. was performed on Kieselgel 60 (Merck) with detection by u.v. light or charring with sulfuric acid. Column chromatography was performed on Kieselgel 60 (Merck, 230–400 mesh) with dichloromethane–methanol–ethanol–water and ethyl acetate–iso-octane (for acetylated compounds) as eluents unless otherwise indicated. The purity was determined by h.p.l.c.[7]. Acetylation was effected conventionally with pyridine–acetic anhydride and deacetylation with methanolic sodium methoxide. Duolite C-6 (H^+) resin (methanol-washed and dried over P_2O_5) was used for neutralisations. The structure of the acetylated glycosides was determined by ^{1}H- and ^{13}C-n.m.r. spectroscopy [Varian XL 200 instrument; internal Me_4Si or sodium 3-(trimethylsilyl)propionate-d_4 (TSP)]. The assignments were based on double resonance and DEPT experiments[7,11,12]. That a linkage was (1→3) was indicated by the marked upfield shift of the H-3 resonance. Similarly, (1→6) linkages were indicated by the marked downfield shift of the C-6 resonance.

Enzymic synthesis with β-D-galactosidase. — (*b*) *Allyl β-D-galactopyranoside* (**1**) *and allyl 3-O-* (**2**) *and 6-O-β-D-galactopyranosyl-β-D-galactopyranoside* (**3**). — To a solution of lactose (100 g) in buffer (300 mL; 0.03M sodium phosphate, mM $MgCl_2$, 5mM 2-mercaptoethanol, pH 7.8) was added allyl alcohol (50 mL) and β-D-galactosidase (2.5 mg, 1550 U) dissolved in buffer (1 mL). The mixture was stored

for 4 days at 37° and the reaction was stopped by heating for 10 min at 70°. The products were isolated by column chromatography on Kieselgel 60 (dichloromethane–methanol–ethanol–water, 6:3.5:1:0.8), which gave **1** (16 g, from 35 fractions, 40 mL) that was pure on the basis of n.m.r. spectroscopy. The material in the remaining fractions (60 × 40 mL) containing **1–3** was acetylated and column chromatography (1:1 toluene–ethyl acetate) then gave the pure acetylated products which were deacetylated to give **1** (4 g), **2** (0.9 g), and **3** (0.3 g).

Compound **1** had m.p. 101–102°; lit.[13] m.p. 101–102°. The n.m.r. data corresponded well with those reported[13].

Compound **2** had m.p. 213–215°, $[\alpha]_D^{21}$ +11° (*c* 1.2, water). N.m.r. data (D_2O): ^1H, δ 6.10–5.90 (m, 1 H, CH=), 5.45–5.28 (m, 2 H, CH_2=), 4.62 (d, 1 H, *J* 7.5 Hz, H-1 or H-1′), 4.51 (d, 1 H, *J* 7.5 Hz, H-1 or H-1′), 4.47–3.57 (m, 14 H); ^{13}C, δ 136.10 (CH=), 121.63 (CH_2=), 107.18, 104.17 (C-1,1′), 85.24 (C-3), 77.90, 77.60, 75.30, 73.84, 73.35, 72.6, 71.40, 71.25 (C-2,2′,3′,4,4′,5,5′, CH_2–CH), 63.76 and 63.70 (C-6,6′).

Anal. Calc. for $C_{15}H_{26}O_{11} \cdot H_2O$: C, 45.00; H, 7.00. Found: C, 45.35; H, 7.16.

Compound **3** had m.p. 134–135°, $[\alpha]_D^{21}$ −14.5° (*c* 1, water). N.m.r. data (D_2O): ^1H, δ 6.08–5.75 (m, 1 H, CH=), 5.43–5.26 (m, 2 H, CH_2=), 4.48–3.48 (m, 16 H); ^{13}C, δ 136.23 (CH=), 121.45 (CH_2=), 106.09, 104.67 (C-1,1′), 77.97, 76.62, 75.52, 75.46, 73.56 (2 C), 73.49, 71.72, 71.45 (2 C, C-2,2′,3,3′,4,4′,5,5′,6 CH_2, CH=), and 63.80 (C-6′). The n.m.r. data for acetylated **3** corresponded well with those reported[14].

Anal. Calc. for $C_{15}H_{26}O_{11} \cdot H_2O$: C, 45.00; H, 7.00. Found: C, 45.10; H, 6.89.

Compound **2** was also synthesised from lactose (5.4 g) and allyl β-D-galactopyranoside (5 g), dissolved in 50 mL of the above buffer containing 1 mg of β-D-galactosidase. The mixture was stored for 48 h at room temperature, and the products were isolated and acetylated as described above. Column chromatography gave pure (n.m.r.) acetylated **2** (720 mg); acetylated **3** was not isolated.

(*b*) *Benzyl β-D-galactopyranoside* (**4**), *benzyl 3-O-* (**5**) *and 6-O-β-D-galactopyranosyl-β-D-galactopyranoside* (**6**). To a solution of lactose (33 g) in 800 mL of buffer (sodium phosphate, mM $MgCl_2$, 5mM 2-mercaptoethanol, pH 7.2) and benzyl alcohol (20 mL) was added β-D-galactosidase (5 mg) dissolved in buffer (1 mL). The mixture was gently agitated for 2 days at room temperature and the reaction was terminated by extraction of the reaction mixture with 2-butanol. Column chromatography (dichloromethane–methanol–ethanol–water, 2.5:0.84:0.19:0.24) gave pure **4** (3.5 g, from 25 fractions, 40 mL) which, after crystallisation from ethyl acetate–2-propanol–water, had m.p. 106°; lit.[15] m.p. 106–107°.

The material in the remaining fractions (40 × 40 mL) containing **4–6** was acetylated and column chromatography then gave pure acetylated **4** (3 g), **5** (0.4 g), and **6** (0.1 g). Deacetylation gave **5** which, after crystallisation from methanol, had m.p. 155–156°; lit.[16] m.p. 162–163°. Compound **6** was pure after deacetylation, $[\alpha]_D^{21}$ −27° (*c* 0.7, 1:1 methanol–water). N.m.r. data (D_2O): ^1H, δ (35°) 7.48–7.40 (m, 5 H, Ph), 5.00–4.73 (q, 2 H, CH_2Ph), 4.58–4.42 (m, 2 H, H-1,1′), 4.10–3.49

(m, 12 H); ^{13}C, δ 139.44, 131.33 (2 C), 131.19 (2 C), 131.01 (aromatic C), 105.91, 104.58 (C-1,1'), 77.78, 76.54, 75.35, 75.28, 74.17, 73.36, 73.32, 71.44, 71.30, 71.23 (C-2,2',3,3',4,4',5,5',6 and CH_2Ph), and 63.61 (C-6').

Anal. Calc. for $C_{19}H_{28}O_{11} \cdot H_2O$: C, 50.67; H, 6.67. Found: C, 50.40; H, 6.49.

Compounds **5** (0.5 g) and **6** (0.13 g) were also synthesised from lactose (3.6 g) and benzyl β-D-galactopyranoside (2.7 g) dissolved in buffer (30 mL) with 2.5 mg of β-D-galactosidase. In another experiment, benzyl β-D-galactopyranoside (0.9 g) was used alone for the synthesis of **5** (0.06 g; 0.1 mg of β-D-galactosidase).

(*c*) *Trimethylsilylethyl β-D-galactopyranoside* (**7**) *and trimethylsilylethyl 3-O-β-D-galactopyranosyl-β-D-galactopyranoside* (**8**). — The same procedure was used as in (*a*) with lactose (12 g) dissolved in 150 mL of buffer, trimethylsilylethanol (10 mL), and β-D-galactosidase (2 mg). The reaction was terminated after 4 days at room temperature and the products were isolated and acetylated by the procedures described above. Deacetylation gave **7** (0.9 g) which, crystallised from iso-octane–ethyl acetate–2-propanol, had m.p. 118–119°, $[\alpha]_D^{21}$ −23° (*c* 1.2, ethanol). N.m.r. data (D_2O): ^1H, δ 4.42 (d, 1 H, *J* 7.7 Hz, H-1), 4.14–3.45 (m, 8 H), 1.12–0.94 (m, 2 H, CH_2Si), 0.048 (Me_3Si); ^{13}C, δ 104.76 (C-1), 77.68, 75.55, 73.37, 71.26, 70.94 (C-2,3,4,5 and OCH_2), 63.48 (C-6), 20.21 (CH_2Si), and 0.145 (Me_3Si).

Anal. Calc. for $C_{11}H_{24}O_6Si \cdot H_2O$: C, 48.18; H, 9.49. Found: C, 47.90; H, 9.15.

Compound **8** had $[\alpha]_D^{21}$ +3.5° (*c* 0.3, 1:3 water–ethanol). N.m.r. data (D_2O): ^1H, δ 4.65 (d, 1 H, *J* 6.7 Hz, H-1 or H-1'), 4.50 (d, 1 H, *J* 7.4 Hz, H-1 or H-1'), 4.22–3.53 (m, 14 H), 1.14–1.00 (m, 2 H, CH_2Si), 0.065 (Me_3Si); ^{13}C, δ 107.01, 104.40 (C-1,1'), 85.22 (C-3), 77.70, 73.33, 73.15, 73.69, 72.48, 71.28, 71.08, 70.93, (C-2,2',3'4,4',5,5' and OCH_2), 63.60, 63.46 (C-6,6'), 20.20 (CH_2Si), and 0.11 (Me_3Si).

Anal. Calc. for $C_{17}H_{34}O_{11}Si \cdot 1.5\ H_2O$: C, 43.50; H, 7.89. Found: C, 43.40; H, 7.62.

Enzymic synthesis with α-D-galactosidase. — (*a*) *Allyl α-D-galactopyranoside* (**9**). To a solution of raffinose (200 g) in buffer (1.5 L, 0.03M sodium phosphate, pH 6.6) and allyl alcohol (150 mL) was added jack-bean meal (200 g; 20 U, 22°, pH 6.6, *p*-nitrophenyl α-D-galactopyranoside). The mixture was stored for 4 days at 40° and then extracted with 2-butanol. Column chromatography (dichloromethane–methanol–ethanol–water, 95:35:10:8) of the material in the extract gave **9** (11 g), m.p. 145–146°; lit.[17] m.p. 145–146°. The n.m.r. data corresponded well with those reported[17].

(*b*) *Allyl 3-O-α-D-galactopyranosyl-α-D-galactopyranoside* (**10**). — To a solution of *p*-nitrophenyl α-D-galactopyranoside (0.9 g) and allyl α-D-galactopyranoside (2 g) in 0.03M sodium phosphate (20 mL, pH 6.5) was added α-D-galactosidase (coffee bean, 0.5 mL, 25 U). The mixture was stored at room temperature and the reaction was monitored spectrophotometrically at 405 nm (nitrophenol). The reaction was terminated after 48 h and, after column chromatography as in (*a*), the product was acetylated. Column chromatography (4:3 toluene–ethyl acetate) then

gave acetylated **10** (0.45 g). Deacetylation and crystallisation from methanol–2-propanol gave **10** (0.21 g), m.p. 160–161°, $[\alpha]_D^{21}$ +230° (*c* 0.5, 1:0.5 water–methanol). N.m.r. data (D_2O): ^1H, δ 6.11–5.92 (m, 1 H, CH=), 5.44–5.26 (m, 2 H, CH_2=), 5.18 (d, 1 H, *J* 3.4 Hz, H-1 or H-1'), 5.07 (s, 1 H, H-1 or H-1'), 4.32–3.75 (m, 14 H); ^{13}C, δ 136.37 (CH=), 120.86 (CH_2=), 100.25, 97.75 (C-1,1'), 76.97 (C-3), 73.75, 73.60, 72.09, 72.04, 71.20, 71.01, 69.42, 68.24 (C-2,2',3'4,4',5,5', CH_2–CH=), and 63.96 (2 C, C-6,6').

Anal. Calc. for $C_{15}H_{26}O_{11} \cdot 0.5\ H_2O$: C, 46.04; H, 6.91. Found: C: 45.95; H, 7.02.

DISCUSSION

The synthesis of useful acceptor glycosides by transglycosidation has been examined using α- and β-D-galactosidase. The cheap oligosaccharides lactose and raffinose were used as glycosyl donors, and allyl alcohol, benzyl alcohol, and trimethylsilylethanol as the acceptors. The products were used, either *in situ* or after isolation, for the synthesis of disaccharide glycosides. Although the syntheses of the disaccharide glycosides were not completely regiospecific, purification of the isomeric products by column chromatography was straightforward since the transglycosidations were stereospecific and no anomerisation of the acceptor glycoside occurred. Thus, the purity of products was usually >99%. The structures of the products were unambiguously determined by n.m.r. spectroscopy (double-resonance, DEPT).

The results are summarised in Table I, which shows that the β-D-galactosidase-catalysed transglycosidations were efficient, allowing accumulation of digalactosides in preparative amounts. Thus, although not optimised, this type of

TABLE I

FORMATION OF MONO- AND DI-SACCHARIDE GLYCOSIDES USING α- AND β-D-GALACTOSIDASE

Glycosyl donor	*Acceptor*	*Main glycosides formed*
α-D-*Galactosidase*		
Raffinose	Allyl alcohol	α-D-Gal-$OCH_2CH{=}CH_2$
α-D-Gal-$OPhNO_2$-*p*	α-D-Gal-$OCH_2CH{=}CH_2$	α-D-Gal-(1→3)-α-D-Gal-$OCH_2CH{=}CH_2$
β-D-*Galactosidase*		
Lactose	Allyl alcohol	β-D-Gal-$OCH_2CH{=}CH_2$ β-D-Gal-(1→3)-β-D-Gal-$OCH_2CH{=}CH_2$ β-D-Gal-(1→6)-β-D-Gal-$OCH_2CH{=}CH_2$
Lactose	Benzyl alcohol	β-D-Gal-OCH_2Ph β-D-Gal-(1→3)-β-D-Gal-OCH_2Ph β-D-Gal-(1→6)-β-D-Gal-OCH_2Ph
Lactose	Trimethylsilylethanol	β-D-Gal-$O(CH_2)_2Si(Me)_3$ β-D-Gal-(1→3)-β-D-Gal-$O(CH_2)_2Si(Me)_3$

reaction was used in one-pot syntheses of 20 g of allyl β-D-galactopyranoside and 0.9 and 0.3 g, respectively, of allyl 3-*O*- and 6-*O*-β-D-galactopyranosyl-β-D-galactopyranoside from 100 g of lactose and 50 mL of allyl alcohol. Only a small amount (2.5 mg) of β-D-galactosidase was required. Similar yields of the corresponding benzyl glycosides were obtained, despite the low concentration of benzyl alcohol (2.5%, v/v).

The syntheses of benzyl glycosides were initially carried out in two-phase systems, consisting of buffer and the alcohol, of the type used to facilitate conversions with other types of enzymes[4]. However, the stability and activity of β-D-galactosidase and the yield of products were higher when the concentration of alcohol was low. Benzyl alcohol is a much more efficient acceptor than water, since the initial yields of transfer (benzyl β-D-galactopyranoside) and hydrolysis (galactose) products were practically the same despite the much higher concentration of water. This finding indicates the presence of hydrophobic regions in the acceptor binding-site of the enzyme and accords with the reported higher affinity of β-D-galactosidase for glycosides with hydrophobic aglycons than for the corresponding sugars[18].

The yield and optimal time of enzymic transglycosidations are a function of the initial concentration of reactants and of the various dissociation and catalytic rate constants for the different steps shown in the scheme[10]. Thus, a high yield may be expected when the rate constant for the formation of the glycosyl–enzyme intermediate from the donor is high compared to that of the product-glycoside. This situation rationalises the low yields in the α-D-galactosidase-catalysed formation of α-D-galactosides from raffinose. Preliminary results show that, when *p*-nitrophenyl α-D-galactopyranoside was used as donor, a higher yield of monogalactosides as well as accumulation of digalactosides occurred.

The data in Table I show that the regioselectivity of the α- and β-D-galactosidase-catalysed formation of digalactosides was the same as previously reported with methyl α- or β-D-galactopyranosides as acceptors[7,8]. Thus, with α-D-galactosidase, the α-(1→3)-linked digalactoside was formed almost exclusively when allyl α-D-galactopyranoside was used as acceptor; with β-D-galactosidase, the β-(1→3)-linked digalactoside preponderated over the (1→6) isomer when the various β-glycosides were used as acceptors. Furthermore, the use of lactose and benzyl β-D-galactopyranoside as donors instead of nitrophenyl β-D-galactopyranoside[7,8] did not affect the regioselectivity.

Because of the simplicity of enzymic synthesis, the yields, especially of the β-linked mono- and di-galactosides obtained in one-pot reactions, make the method attractive. Furthermore, crude and small amounts of enzyme preparations may be used. The re-utilisation of enzymes is facilitated by their immobilisation[7,19].

The disaccharide glycosides synthesised above occur widely in various glycoconjugates. Thus, α- and β-D-Gal-(1→3)-D-Gal are structural units of several types of glycolipids[2]. The allyl[20], benzyl[16], and trimethylsilylethyl[21] groups are frequently used for temporary protection of the anomeric centre, and α-D-Gal-(1→3)-D-Gal, β-D-Gal-(1→3)-D-Gal, and β-D-Gal-(1→6)-D-Gal may thus be obtained. In addi-

tion, benzyl glycosides can be used as inhibitors[22], and allyl glycosides can be used for the preparation of synthetic antigens or affinity adsorbents[13] and for the construction of spacer arms[23,24] and affinity labels[14] after chemical modification.

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