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Enzymic synthesis of HexNAc-containing disaccharide glycosides*

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ABSTRACT

The following disaccharide glycosides were obtained from the appropriate donor and acceptor glycosides by employing glycosidases from the mollusc *Chamelea gallina* as catalysts: α-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-OMe (*N*-acetyl-α-D-galactosaminidase), β-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-OMe, β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-OMe, and β-D-Glc*p*NAc-(1→6)-α-D-Man*p*-OMe (*N*-acetyl-β-D-hexosaminidase). The regioselectivity of the *N*-acetyl-β-D-hexosaminidase-catalysed reactions depended on the anomeric configuration of the acceptor. Thus, α-D-Gal*p*-OMe gave β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*-OMe almost exclusively, whereas β-D-Galp-OMe gave β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-OMe and β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-OMe in almost equal amounts. The isolation of the products by chromatography was straightforward.

INTRODUCTION

Glycosidases have long been used as analytical tools in glycoconjugate research and are being applied increasingly for selective synthesis of complex carbohydrate structures[1]. Methods for the syntheses of such structures in amounts sufficient for further study are important. Chemical syntheses usually require multi-step procedures[2] and syntheses with enzymes are attractive because of their stereospecificity and regioselectivity. Thus, several of the common disaccharide sequences in glycoconjugates can now be prepared[1,3] conveniently, by transglycosylation using a glycosidase–donor–acceptor system.

The use of glycosides as acceptors gives products that can be utilised variously[1,3,4], and simplifies purification since anomerisation is prevented. Moreover, the regioselectivity of glycosidases can be manipulated by changing the structure of the aglycon or the anomeric configuration of the acceptor glycoside[3]. Syntheses of trisaccharides and higher oligosaccharides have been achieved by employing readily available glycosidases for the synthesis of shorter saccharides, and glycosyltransferases for catalysis of the final steps when demands on regioselectivity are higher[5].

Molluscs contain high levels of glycosidase activities[6] and the synthesis is now reported of glycosides of the type HexNAc–Hex, which act as receptors for pathogens[7]

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and are part of several antigenic determinants[8], using a glycosidase preparation obtained from the mollusc *Chamelea gallina*.

EXPERIMENTAL

General. — The mollusc was obtained at a local shop in southern Spain. The livers were isolated, homogenised, and extracted with distilled water, and the material in the supernatant solution was fractionated[6] with ammonium sulphate. The precipitate was used without further purification.

The general and instrumental methods were as described[3,4,9]. The reactions were followed by measuring the amount of liberated *p*-nitrophenol (405 nm) or by h.p.l.c. (LKB equipment; Merck Lichrosorb NH_2-column; aqueous 70% acetonitrile). 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 by charring with sulfuric acid. Column chromatography was performed on Sephadex G-10 (Pharmacia) followed by semi-preparative h.p.l.c. (Lichrosorb NH_2-column), using water (Sephadex) or 7:3 acetonitrile–water (h.p.l.c.).

Acetylation was effected conventionally with pyridine–acetic anhydride and the products were deacetylated with methanolic sodium methoxide.

The structure of the acetylated glycosides was determined by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Varian XL 300 instrument). The assignments were based on double-resonance and DEPT-experiments[3,4,9]. (1→3) Linkages were indicated by the marked upfield shift of the H-3 resonance, and (1→6) linkages by the marked downfield shift of the C-6 resonance.

Methyl 6-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-galactopyranoside (**1**). — To a mixture of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (0.34 g), methyl α-D-galactopyranoside (2.0 g), sodium citrate/phosphate buffer (19 mL, pH 5.8), and *N,N*-dimethylformamide (1 mL) was added ammonium sulphate precipitate[6] (100 mg) that contained *N*-acetyl-β-D-glucosaminidase. The mixture was agitated gently at room temperature. After 65 h, the reaction was stopped by heating for 5 min at 80°. Column chromatography (Sephadex G10, semi-preparative h.p.l.c.) gave **1** (33 mg), m.p. 191° (from ethanol), $[\alpha]_D$ +45° (*c* 0.4, water). N.m.r. data (D_2O): ^{1}H, δ 4.80 (d, 1 H, *J* 2.7 Hz, H-1), 4.53 (d, 1 H, *J* 8.4 Hz, H-1'), 4.03–3.41 (m), 3.37 (s, 3 H, OMe), 2.01 (s, 3 H, NAc); ^{13}C, δ 178.88 (C=O), 104.25, 102.02 (C-1,1'), 78.54, 76.43, 72.62, 72.03, 71.97 (2 C), 70.80 (C-2,3,3',4,4',5,5'), 72.15 (C-6), 63.40 (C-6'), 58.19, 57.55 (C-2', OCH_3), 24.78 ($NCOCH_3$).

Anal. Calc. for $C_{15}H_{27}NO_{11}\cdot H_2O$: C, 43.37; H, 6.98; N, 3.37. Found: C, 43.10; H, 6.50; N, 3.40.

In a similar experiment with the same donor (0.68 g), but with methyl α-D-mannopyranoside (2.0 g) as acceptor, methyl 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside (45 mg, purity >95% according to n.m.r. and h.p.l.c.) was obtained. The ^{1}H- and ^{13}C-n.m.r. data corresponded with those reported[10].

Methyl 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside

(**2**). — To a solution of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (0.34 g) and methyl β-D-galactopyranoside (2.0 g) in 0.03M sodium phosphate/citrate buffer (20 mL, pH 5.8) was added ammonium sulphate precipitate (100 mg). After storage with gentle agitation for 80 h at 37°, the mixture was processed, as described above, to give **2** (15 mg), m.p. 255° (from ethanol), $[\alpha]_D$ −4° (*c* 0.4, water); lit.[11] m.p. 256–257°, $[\alpha]_D$ −3.3° (water). The n.m.r. data corresponded with those reported[11].

A separate fraction (20 mg), which was not further purified, contained the β-(1→6)-linked isomer (purity 75% according to n.m.r.) and an unidentified disaccharide methyl glycoside.

Methyl 3-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-β-D-galactopyranoside (**3**). — To a solution of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (0.50 g) and methyl β-D-galactopyranoside (2.5 g) in 0.04M sodium phosphate buffer (20 mL, pH 5.8) was added ammonium sulphate precipitate (100 mg). After storage with gentle agitation for 120 h at 37°, the mixture was processed, as described above, to give **3** (36 mg), m.p. 197° (from ethanol), $[\alpha]_D$ +27° (*c* 0.5, water). N.m.r. data (D_2O): ^1H, δ 4.61 (d, 1 H, *J* 8.3 Hz, H-1'), 4.28 (d, 1 H, *J* 7.9 Hz, H-1), 4.12 (d, 1 H, *J* 3.2 Hz, H-4), 3.54 (s, 3 H, OMe), 2.01 (s, 3 H, NAc); ^{13}C, δ 177.91 (C=O), 106.61, 105.87 (C-1,1'), 84.73 (C-3), 77.71, 77.40, 73.50, 72.53, 71.19, 70.48 (C-2,3',4,4',5,5'), 63.71, 63.63, (C-6,6'), 59.90, 55.35 (OCH_3, C-2'), 24.97 ($NCOCH_3$).

Anal. Calc. for $C_{15}H_{27}NO_{11}\cdot 1.5H_2O$: C, 42.45; H, 7.03; N, 3.30. Found: C, 42.50; H, 6.70; N,3.20.

In a similar experiment with *o*-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (70 mg) and methyl α-D-galactopyranoside (1.0 g) as substrates, methyl 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-α-D-galactopyranoside (5 mg, purity according to n.m.r. >95%) was obtained. N.m.r. data (D_2O): ^1H, δ 5.04 (d, 1 H, *J* 3.7 Hz, H-1'), 4.87 (d, 1 H, *J* 3.4 Hz, H-1), 4.13 (d, 1 H, *J* 3.6 Hz, H-4), 3.41 (s, 3 H, OMe), 2.04 (s, 1 H, NAc); ^{13}C, δ 177.36 (C=O), 102.01, 96.30 (C-1,1'), 76.50 (C-3), 73.66, 73.41, 71.15, 70.33, 69.35, 68.05 (C-2,3',4,4',5,5'), 63.99, 63.75 (C-6,6'), 57.77, 52.38 (OCH_3, C-2'), 24.97 ($NCOCH_3$).

RESULTS AND DISCUSSION

The glycosidase-containing preparation obtained from the liver of *C. gallina* catalysed the stereospecific synthesis of the disaccharide glycosides shown in Table I. A crude precipitate that contained various glycosidases was used in the reactions. Nitrophenyl glycosides were chosen as donors since they were good substrates for the glycosidases and side reactions involving hydrolysis or transglycosylation of the acceptor methyl glycosides catalysed by contaminating glycosidases were minimised. The reactions could be monitored by spectrophotometry of the nitrophenol liberated.

The syntheses of β-GalNAc-(1→3)-β-Gal-OMe, β-GlcNAc-(1→6)-α-Gal-OMe, and β-GlcNAc-(1→6)-α-Man-OMe were highly regioselective. This finding, together with the stereospecific transglycosylations and the use of glycosides as acceptors, facilitated column chromatography of the products.

TABLE I

Formation of disaccharide glycosides using glycosidases from *Chamelea gallina*

Donor	*Acceptor*	*Main product(s)*
β-GlcNAc-OPhNO$_2$-*p*	α-Man-OMe	β-GlcNAc-(1→6)-α-Man-OMe
	α-Gal-OMe	β-GlcNAc-(1→6)-α-Gal-OMe
	β-Gal-OMe	β-GlcNAc-(1→3)-β-Gal-OMe
		β-GlcNAc-(1→6)-β-Gal-OMe
β-GalNAc-OPhNO$_2$-*p*	β-Gal-OMe	β-GalNAc-(1→3)-β-Gal-OMe
α-GlcNAc-OPhNO$_2$-*o*	α-Gal-OMe	α-GlcNAc-(1→3)-α-Gal-OMe

The anomeric configuration of the acceptor can influence the regioselectivity of glycosidase-catalysed transglycosylations[3]. This effect was also observed in the reactions with β-D-Glc*p*NAc-OPhNO$_2$-*p* as donor and with β-D-Gal*p*-OMe or α-D-Gal*p*-OMe as acceptor (Table I). Thus with the α-glycoside, >85% of β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*-OMe was formed whereas, with the β-glycoside as acceptor, the β-(1→6)- and the β-(1→3)-linked isomers were formed in practically equal amounts. The separation of the isomers was straightforward. The regioselectivity of the latter reactions also depends on the source of the enzyme. Thus, jack-bean *N*-acetyl-β-D-glycosaminidase almost exclusively gave[5] β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-OMe.

It was not determined if the syntheses of the glycosides of β-D-GalNAc and β-D-GlcNAc were catalysed by the same glycosidase, but hexosaminidases active on both types of glycosides are known.

The type of donor glycoside employed may influence the yield and regioselectivity of enzymic transglycosylatons[12]. It was found that the yield of α-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-OMe was higher with the more reactive donor α-D-Gal*p*NAc-OPhNO$_2$-*o* than with the corresponding phenyl glycoside. The use of the phenyl glycoside also resulted in a lower regioselectivity [the α-(1→6)- and α-(1→3)-linked isomers were formed in about equal amounts].

The simplicity of the enzymic synthesis and the possibility of using the crude enzyme preparation for the synthesis of various structures make the method attractive.

Recovery of the excess of acceptor glycosides (which are used in high concentration to increase the transglycosylation/hydrolysis ratio) by column chromatography is straightforward. The reactions have not been optimised, but the yields (5–10% of the donor substrate) obtained in this study are comparable with those reported for enzymic synthesis of similar HexNAc-containing disaccharide glycosides[5].

Enzymic synthesis of other types of disaccharide glycosides (*e.g.*, digalactosides, dimannosides, Gal-HexNAc-OR) usually give yields in the range 25–45% (refs. 5, 12, and 13). In these reactions, the yields were increased by changing the reaction conditions [12,13] and preliminary experiments show that up to 50% yields of HexNAc-Gal glcyosides can be obtained by using a high acceptor concentration (0.5–1.0 g/ml buffer).

The disaccharide structures synthesised above occur widely in glycoconjugates. Thus, β-D-GlcNAc-(1→6)-β-D-Gal and β-D-GlcNAc-(1→6)-α-D-Man are present in O-

and N-glycoproteins, respectively[14,15]; increased β-D-GlcNAc-(1→6)-α-D-Man-branching may be associated with metastasis[16]; α-D-GalNAc-(1→3)-D-Gal is part of blood group A substance; β-D-GalNAc-(1→3)-D-Gal is characteristic of the globo-series[17] (one of the major types of glycolipid structures) and is the terminal unit of blood group P; β-D-GlcNAc-(1→3)-D-Gal is characteristic of the lacto-series of glycolipids[17], is part of the repeating unit of the i-antigen[18], acts as a receptor for pathogen[7] (*Streptococcus pneumoniae*), and is a constituent of tumor-associated antigens, as is β-D-GalNAc-(1→3)-D-Gal[8].

The mollusc preparation can be used for selective synthesis of other types of disaccharide sequences. For example, α-Fuc-(1→6)-β-Gal-OMe was prepared in good yield (25%) and with high regioselectivity from α-Fuc-OPhNO$_2$-*p* and β-Gal-OMe[19]. Formation of trisaccharides with exo-glycosidases has been achieved [*e.g.*, α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-OEt[1], β-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-OEtSiMe$_3$[5], α-Man-(1→2)-α-Man-(1→2)-α-Man-OMe[3]]. The possibility of using the mollusc preparation for synthesis of higher oligosaccharides is being investigated.

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