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Note

Chemoenzymatic synthesis of the Thomsen–Friedenreich antigen determinant

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Abstract

The efficient chemoenzymatic synthesis of the Thomsen–Friedenreich antigen determinant is demonstrated under transglycosylation conditions employing β-galactosidase from bovine testes.

Keywords: Chemoenzymatic synthesis; Transglycosylation; β-Galactosidase from bovine testes; Thomsen–Friedenreich antigen determinant

1. Introduction

The enzymatic cleavage of *N*-acetylneuraminic acid from sialoglycoproteins of the erythrocyte membrane causes exposure of the Thomsen–Friedenreich (T) cryptantigen, Galβ1–3GalNAcα1-. The T-antigen is known as a tumor-associated antigen because its appearance in tumor cells has been detected [1]. When coupled to a protein it is suitable for use as an artificial antigen. In recent years, some elegant methods for the glycosylation of the T-antigen determinant have been developed, however these procedures comprise many reaction steps, complicated protecting group chemistry and laborious chromatographic purification [2–4].

As most of the complex hetero-oligosaccharides of interest are produced in nature by enzymes, it is entirely plausible to also use the synthetic potential of enzymes in vitro. Instead of multistep chemical reactions, the synthetic process would be expected to work regio- and stereo-specifically [5]. Our interest in this work has been focused on a synergistic interaction between chemical and chemoenzymatic carbohydrate synthesis which allows the efficient preparation of T-antigen determinants combining the advantage of highly developed chemical synthesis with the selectivity of enzymatic transfer of carbohydrates.

2. Results and discussion

The acceptor derivatives **2a** and **2b** were synthesized employing either classical chemistry or enzymatic condensation conditions (Scheme 1). Enzymatic synthesis was observed employing L-serine or L-threonine and *N*-acetyl-D-galactosamine by inverting the hydrolytic activity of α-*N*-acetylgalactosaminidase isolated from beef liver (EC 3.2.1.49) [6,7]. In contrast, by transglycosylation with activated substrates products could not be obtained. The main disadvantage of this reaction, in terms of glycoside synthesis, is the relatively low yield and the complex

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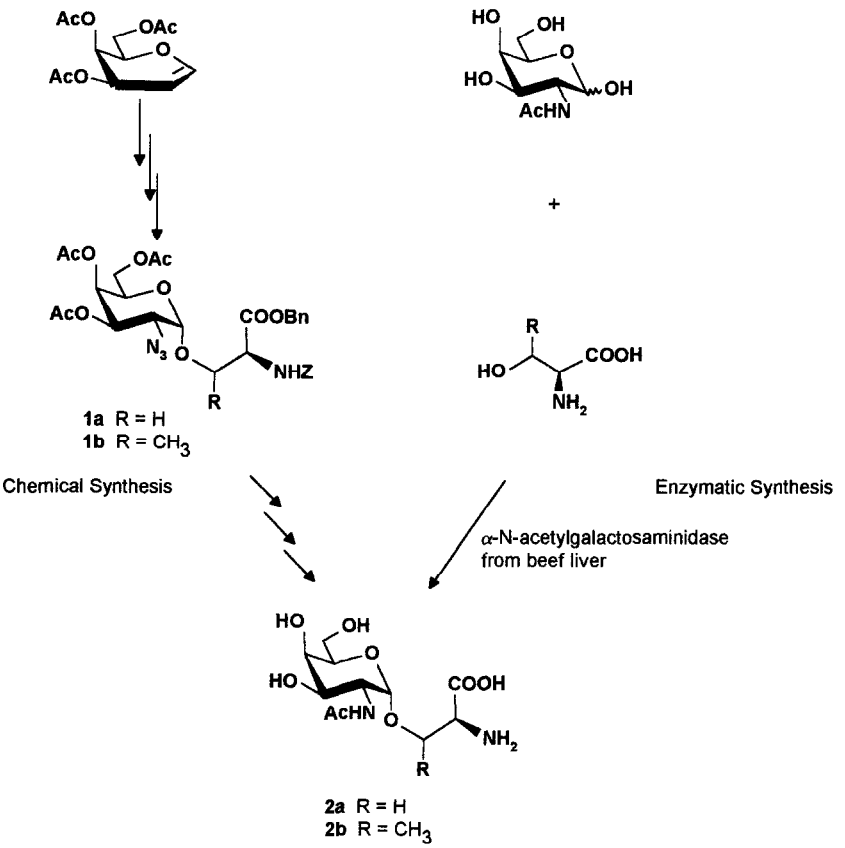


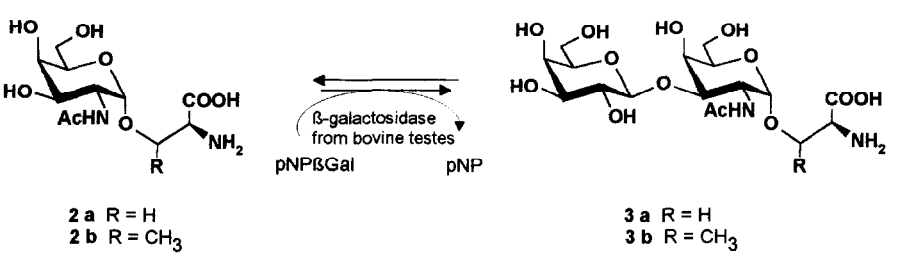
Scheme 1. Synthesis of the monosaccharide amino acid substrate.

mixture of products due to the low specificity of this glycosidase.

Therefore, in this case a more accessible route to the desired product would be by chemical means. Azidonitration conditions [8] were employed to tri-*O*-acetyl-galactal followed by trichloro-acetimidate chemistry [9]. The crucial glycosylations of the amino acid derivatives were realised by using the β-imidate in the presence of trimethylsilyl triflate, to give the α-glycosides **1a** and **1b**. Reduction of the azides applying nickel chloride and sodium borohydride [10] and subsequent acetylation of the products gave the acetamido derivatives. These precursors were initially hydrogenolysed using palladium-carbon as catalyst, followed by methanolic sodium hydroxide to afford the target compounds **2a** and **2b** in a preparative scale.

The synthesis of the T-antigen determinants **3a** and **3b** is based on the transgalactosylation of **2a** and **2b** using β-galactosidase from bovine testes (EC 3.2.1.23) (Scheme 2) [11].

The bovine testes enzyme is commercially available but too expensive to be used in large scale chemistry. A simplified purification procedure [12] was performed which followed the initial steps described by Distler and Jourdian [13]. Working with



Scheme 2. Galactosylation employing β-galactosidase from bovine testes.

crude enzyme preparation can prove to be problematic, for example there is always a risk of destroying enzymes due to the presence of proteases, and there may be undesirable reactions due to other contaminant enzymes. Whereas in the crude enzyme mixture hydrolysis of GalNAcβ1-OR derivatives was observed due to contamination with β-hexosaminidase, no contamination with α-hexosaminidase could be detected.

It is known that β-galactosidase from bovine testes hydrolyses (1–3)-, (1–4)- and (1–6)-linkages of *N*-acetyllactosamine derivatives. In the enzymatic synthesis of the T-antigen determinants the equilibrium was shifted to result in a (1 → 3)- interglycosidic linkage by reversing the hydrolytic activity of this galactosidase and gave **3a** (22%) and **3b** (28%). Formation of (1–4)- or (1–6)-linkages was not observed under these conditions. *p*-Nitrophenyl β-galactopyranoside (*p*NPβGal) was used instead of lactose as the donor to simplify the purification procedure, and, furthermore, to raise the rate of galactose released [14].

A new enzyme, cloned from the genes coding for β-galactosidase from *B. circulans* into *E. coli*, also hydrolyzed the (1–3)-linkage specifically and was applied for the synthesis of one T-antigen determinant, Galβ1–3GalNAcα1-O-Ser [15]. However, the yield (10%) was relatively low in comparison with the bovine testicular β-galactosidase catalyzed reaction.

3. Experimental

Enzyme assay.—The rate of hydrolysis of the substrate *p*-nitrophenyl β-D-galactopyranoside is determined by measuring the absorbance of the liberated nitrophenol in alkaline solutions described by Kuby and Lardy [16]. Incubation mixtures contained the following components in total volumes of 0.1 mL: sodium phosphate-citrate buffer, pH 4.3, as prepared by McIlvaine, 25 μL; *p*-nitrophenyl β-Gal, 0.5 μmole, and 1 to 4 units of enzyme. Control tubes contained the same components but lacked either substrate or enzyme. Incubations were conducted for 30 min at 37 °C and were terminated by the addition of 1 mL of 0.25 M glycine buffer, pH 10. Absorbance was measured in cells with a 1 cm light path at 400 nm.

One Unit (U) is defined as the hydrolysis of 1 mol of *p*-nitrophenyl β-D-galactopyranoside/min under the above conditions.

Protein determination.—Protein concentrations were determined by the method of Bradford [17].

Purification procedure.—All manipulations were performed at 0–4 °C unless otherwise stated.

Bovine testes (210 g) were obtained from a slaughterhouse and stored at −20 °C until use. The testes were thawed and homogenised with a blender. 0.1 M Acetic acid (210 mL) was added, and the pH was adjusted to 4.0 by dropwise addition of 2 M HCl. The homogenate was stirred and centrifuged for 20 min at 10,000 g. Ammonium sulfate was added to the crude extract to 40% saturation and after stirring for 1 h, the precipitate was collected by centrifugation for 20 min at 10,000 g and dissolved in acetate buffer, pH 4.3. The solution was incubated at 50 °C for 15 min before centrifugation for 10 min at 20,000 g. The supernatant solution was dialysed against acetate buffer, pH 4.3 overnight and lyophilised. The specific activity was 30 mU/mg, and the total activity was 31 U.

O-*(3,4,6-Tri*-O-*acetyl*-*2*-*azido*-*2*-*deoxy*-α-D-*galactopyranosyl)*-N-*benzyloxycarbonyl*-L-*serine benzylester* (**1a**).—To a mixture of *O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl)trichloro-acetimidate [9] (95 mg, 0.2 mmol) and *N*-Cbz-L-serine benzyl ester (56 mg, 0.17 mmol) in dichloromethane (6 mL) was added dropwise $Me_3SiOSO_2CF_3$ (4 μL, 0.023 mmol) at −15 °C under argon. After stirring for 30 min at room temperature, the mixture was neutralized with triethylamine and evaporated in vacuo, and the residue chromatographed on SiO_2 in 1:1 petrolether:EtOAc + 1% triethylamine, to give the α-glycoside (69 mg, 54%); $[\alpha]_D$ +70° (c 1.0, chloroform); R_f 0.3 in 1:1 petrolether:EtOAc + 1% triethylamine; ^{1}H NMR data (400 MHz, $CDCl_3$): δ 7.41–7.11 (m, 10 H, Ph), 5.84 (d, 1 H, $J_{CH,NH}$ 8.2, NH), 5.35 (dd, 1 H, $J_{3,4}$ 3.1, H-4), 5.18–5.05 (m, 5 H, 2 $PhCH_2$, H-3), 4.87 (d, 1 H, $J_{1,2}$ 3.6, H-1), 4.59 (m, 1 H, SerαCH), 4.05 (dd, 1 H, $J_{\beta CHa,\beta CHb}$ 10.7, SerβCH), 3.98–3.83 (m, 4 H, 5 H, 6a H, 6b H, SerβCH), 3.45 (dd, 1 H, $J_{2,3}$ 11.2, H-2), 2.10 (s, 3 H, CH_3CO), 2.03 (s, 3 H, CH_3CO), 1.98 (s, 3 H, CH_3CO).

O-*(3,4,6-Tri*-O-*acetyl*-*2*-*azido*-*2*-*deoxy*-α-D-*galactopyranosyl)*-N-*benzyloxycarbonyl*-L-*threonine benzylester* (**1b**).—To a mixture of *O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl)trichloro-acetimidate [9] (1.0 g, 2.1 mmol) and *N*-Cbz-L-threonine benzyl ester (609 mg, 1.77 mmol) in dichloromethane (6 mL) was added dropwise $Me_3SiOSO_2CF_3$ (43 μl, 0.25 mmol) at −15 °C under argon. After stirring for 30 min at room tem-

perature, the mixture was neutralized with triethylamine and evaporated in vacuo, and the residue chromatographed on SiO_2 in 1:1 petrolether:EtOAc + 1% triethylamine, to give the α-glycoside (1.16 g, 84%); $[\alpha]_D$ +67° (c 1.0, chloroform); R_f 0.3 in 1:1 petrolether:EtOAc + 1% triethylamine; ^{1}H NMR data (400 MHz, $CDCl_3$): δ 7.36–7.16 (m, 10 H, Ph), 5.56 (d, 1 H, $J_{CH,NH}$ 9.5, NH), 5.31 (dd, 1 H, $J_{3,4}$ 3.1, H-4), 5.12–5.09 (m, 5 H, 2 $PhCH_2$, H-3), 4.79 (d, 1 H, $J_{1,2}$ 3.6, 1 H), 4.39 (d, 1 H, $J_{\alpha CH,\beta CH}$ 2.0, Thrα CH), 4.34 (dd, 1 H, $J_{\beta CH,CH3}$ 6.4, Thrβ CH), 4.10 (dd, 1 H, $J_{5,6}$ 6.1, H-5), 3.99–3.90 (m, 2 H, 6a H, 6b H), 3.49 (dd, 1 H, $J_{2,3}$ 11.2, H-2), 2.03 (s, 3 H, CH_3CO), 1.92 (s, 3 H, CH_3CO), 1.90 (s, 3 H, CH_3CO), 1.22 (d, 3 H, $J_{\beta CH,CH3}$ 6.4, $ThrCH_3$).

Transglycosylation to give O-*[2-Acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-serine* (**3a**).—*O*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-L-serine (**2a**) [2] (50 mg, 0.162 mmol) and *p*-nitrophenyl β-D-galactopyranoside (72 mg, 0.24 mmol) were dissolved in 50 mM sodium phosphate-citrate buffer, pH 4.3 (1 mL). The reaction mixture was incubated with β-galactosidase from bovine testes (17 mg, 0.5 U) at 37 °C for 48 h. The reaction was terminated by heating at 90 °C for 5 min. The desired product was isolated from a Biogel P2 column with water to give (13 mg, 0.028 mmol); *O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-serine (10 mg, 0.032 mmol) was reisolated; based on the reacted monosaccharide the yield was 22%; $[\alpha]_D$ +35° (c 1.0, water), lit. $[\alpha]_D$ +84° (c 1.2, water) [2]; R_f 0.35 in 7:3 1-propanol:water; ^{1}H NMR data (400 MHz, D_2O): δ 4.87 (d, 1 H, $J_{1,2}$ 3.5, H-1), 4.45 (d, 1 H, $J_{1,2}$ 7.6, H-1′), 4.33 (dd, 1 H, $J_{2,3}$ 11.2, H-2), 4.21 (d, 1 H, $J_{4,5}$ 0.5, H-4), 4.16–3.97 (m, 3 H, H-3, H-5, Serα CH), 3.88 (dd, 1 H, $J_{3,4}$ 3.6, H-4′), 3.78–3.72 (m, 6 H, H-6a, H-6b, H-6a′, H-6b′, Serβ CH_2), 3.66 (ddd, 1 H, H-5′), 3.59 (dd, 1 H, $J_{3,4}$ 3.6, H-3′), 3.56 (dd, 1 H, $J_{2,3}$ 10.2, H-2′), 2.00 (s, 3 H, CH_3CO); ^{13}C NMR (250 MHz, D_2O): δ 175.03, 160.69 (C=O), 105.02 (C-1′), 98.41 (C-1), 77.09 (C-3), 75.31 (C-5′), 72.84 (C-3′), 71.39 (C-5), 70.93 (C-2′), 69.05 (C-4), 68.92 (C-4′), 68.36 (Serβ CH), 61.58 (C-6), 61.34 (C-6′), 55.14 (Serα CH), 48.77 (C-2), 22.43 ($NCOCH_3$); $C_{17}H_{30}O_{13}N_2$ (470.43) *m/z* 469.

O-*[2-Acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine* (**3b**).—*O*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine (**2b**) [2] (50 mg, 0.155 mmol) and *p*-nitrophenyl β-D-galactopyranoside (72 mg, 0.24 mmol) were dissolved in 50 mM sodium phosphate-citrate buffer, pH 4.3 (1 mL). The reaction mixture was incubated with β-galactosidase from bovine testes (17 mg, 0.5 U) at 37 °C for 48 h. The reaction was terminated by heating at 90 °C for 5 min. The desired product was isolated from a Biogel P2 column with water to give (16 mg, 0.035 mmol); *O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine (10 mg, 0.032 mmol) was reisolated; based on the reacted monosaccharide the yield was 28%; $[\alpha]_D$ +72° (c 1.0, water), lit. $[\alpha]_D$ +90° (c 1.4, water) [2]; R_f 0.35 in 7:3 1-propanol:water; ^{1}H NMR data (400 MHz, D_2O): δ 4.89 (d, 1 H, $J_{1,2}$ 3.6, H-1), 4.41 (d, 1 H, $J_{1,2}$ 7.6, H-1′), 4.37 (m, 1 H, Thrβ CH), 4.22 (dd, 1 H, $J_{2,3}$ 10.9, H-2), 4.06 (d, 1 H, $J_{3,4}$ 2.5, H-4), 3.96 (m, 1 H, H-5), 3.91 (dd, 1 H, $J_{3,4}$ 2.5, H-3), 3.84 (d, 1 H, $J_{3,4}$ 3.1, H-4′), 3.69–3.66 (m, 5 H, H-6a, H-6b, H-6a′, H-6b′, Thrα CH), 3.58 (m, 1 H, H-5′), 3.56 (dd, 1 H, $J_{3,4}$ 3.6, H-3′), 3.43 (dd, 1 H, $J_{2,3}$ 10.2, H-2′), 1.97 (s, 3 H, CH_3CO), 1.35 (d, 3 H, $J\beta_{CH,CH3}$ 6.6, $ThrCH_3$); ^{13}C NMR (250 MHz, D_2O): δ 104.98 (C-1′), 99.70 (C-1), 77.19 (C-3), 75.40 (C-5′), 74.98 (Thrβ CH), 72.91 (C-3′), 71.04 (C-5), 71.62 (C-2′), 69.17 (C-4), 68.01 (C-4′), 61.64 (C-6), 61.43 (C-6′), 59.48 (ThrCH), 48.97 (C-2), 22.76 ($NCOCH_3$), 18.68 ($ThrCH_3$); $C_{18}H_{32}O_{13}N_2$ (484.55) *m/z* 483.

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