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Enzymatic galactosylation of *C*-glycosides analogues en route to *C*-glycopeptides

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1. Introduction

The synthesis of glycoproteins and glycopeptides, as well as the elucidation of their biological functions are among the key issues of modern biochemistry and organic chemistry, as it has been documented in recent reviews focused on the biochemical process of protein glycosylation [1], on the various types of glycosidic linkages that are present in natural glycopeptides and glycoproteins [2], and on the synthesis of glycopeptides [3].

Enzymes have also been exploited to support these synthetic efforts. Specifically, hydrolases (lipases, proteases and acylases) have been used for the mild and selective deprotection of amino acids and for the coupling of *N*- and *O*-glycopeptides fragments [4,5], while glycosyltransferases have been used to build new oligosaccharides units on glycopeptides [6] and even on the small protein Ribonuclease B [7].

The synthesis or the manipulation of glycopeptides is frequently hampered by the sensitivity of the glycosidic linkage between the (oligo)saccharide and the peptide chain towards both chemical and enzymatic hydrolysis. A way to overcome this problem is to incorporate *C*-glycosides (instead of *O*-glycosides) into the peptide chain obtaining the so-called "glycopeptidomimetics", compounds that emulate the natural compounds but have enhanced stability [2]. However, despite the importance of these compounds, very few reports have appeared on the chemical and/or enzymatic elaboration of their pseudosugar moieties to get more complex carbohydrates. In a previous paper [8], we have shown, for the first time, that the hydroxymethyl derivative of glucose, **1**, as well its *N*-protected benzyloxycarbonyl analogue **2** are excellent substrates for the β-1,4-galactosyltransferase from bovine colostrum (GalT), affording the corresponding pseudo-lactosides **1a** and **2a** in good yields. This communication will present the results obtained with the *C*-glucoside **3**, analogue

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of a β-D-glucopyranosyl serine, and an extension of our investigation to *C*-analogues of *N*-acetyl-D-glucosamine.

2. Experimental

2.1. Materials and methods

UDP-glucose, α-lactalbumin from bovine milk, UDP-galactose-4′-epimerase (EC 5.1.3.2, from galactose-adapted yeast) and alkaline phosphatase (EC 3.1.3.1, from bovine intestinal mucose, type VII S) were from Sigma. β-1,4-Galactosyltransferase (EC 2.4.1.22, from bovine colostrum) was purified as described elsewhere [9] and its activity was evaluated with by a spectrophotometric assay [10]. Capillary electrophoresis separations were performed in a Spectraphoresis 1000 capillary system Thermo Separation Products; data collection was performed on a personal computer utilizing SW-Phoresis 1000 software; in all the experiments, fused-silica capillaries (Polymicro Technologies) 58 cm (50 cm to the window) × 50 μm i.d. were used; sugar samples were detected at 254 nm; before each run, the capillary was washed with 0.1 M NaOH for 2 min and with deionized water for 2 min. Melting points were determined using a Kofler apparatus and are uncorrected. Optical rotations were measured using a Perkin-Elmer 141 polarimeter. TLC: precoated silica gel 60 F_{254} plates (*E. Merck*). Flash chromatography: silica gel 60 (70–230 mesh, *E. Merck*).

2.2. Synthesis of substrates *3–5*

Compound **3** was synthesized as already reported [11]. Compound **4** was purchased from Aldrich. Compound **5** was prepared starting from *N*-acetyl-D-glucosamine and nitromethane as suggested in Ref. [12]. The intermediate 2-acetamido-2-deoxy-β-D- glucopyranosyl - nitromethane (1 g, 4.46 mmol) was dissolved in a mixture of methanol (15 ml) and water (6 ml). To this solution, Pd-OH/C 10% (100 mg) and HCl 37% (360 μl) were added and the mixture was stirred under H_2 atmosphere at ambient pressure for 36 h. The catalyst was removed by filtration and the solvent was evaporated to give 1.01 g of 3-acetamido-2,6-anhydro-1-amino-1,3-dideoxy-D-*glycero*-D-*gulo*-heptitol hydrochloride (quant.), which was directly used for the next step. 3-Acetamido-2,6-anhydro-1-amino-1,3-dideoxy-D-*glycero*-D-*gulo*-heptitol hydrochloride (1 g, 4.35 mmol) was dissolved in a solution of $NaHCO_3$ (1.1 g, 13.05 mmol) in 10 ml of water. Under vigorous stirring, CbzCl (0.92 ml, 6.525 mmol) was then added in four portions over 1 h and the mixture was stirred overnight. The solution was extracted repeatedly with ether and the aqueous phase evaporated. The residue was purified by flash chromatography (AcOEt–MeOH 7:3) to give 1.35 g (95%) of **5**. Amorphous solid. ^{1}H NMR (D_2O, 50°C) δ 7.55 (m, 5H, ArH), 5.26 (br s, 2H, CH_2Ph), 4.1–3.3 (m, 9H, H-1, H-2, H-3, H-4, H-5, H-6, H-7) 2.18 (s, 3H, CH_3); ^{13}C NMR (75 MHz, D_2O) δ 175.3 (s, CH_3C=O) 159.1 (s, N–C=O), 137.4 (s, Ar), 129.7, 129.3, 128.6 (3s, 5 Ar C), 80.3 (d), 77.9 (d), 76.0 (d), 71.0 (d), 67.9 (t, CH$_2$Ph), 61.9 (t, C-7), 53.6 (d, C-3), 42.6 (t, CH$_2$N), 23.0 (q, CH$_3$).

2.3. Methyl 8-O-(β-D-galactopyranosyl)-5,9-anhydro-2-carbobenzyloxyamino-2,3,4-trideoxy-D-eryitro-L-galacto-decuronate (*3a*)

A 6.4-ml solution of 50 mM Tris buffer pH 7.4 containing **3** (64 mg, 0.155 mmol, 20 mM), UDP-glucose (488 mg, 0.775 mmol, 100 mM), $MnCl_2$ (25 mM), 0.5 U/ml GalT, 2 U/ml epimerase, 3.5 U/ml alkaline phosphatase, 1 mg/ml α-lactalbumin, dithiothreitol (1 mM) and NaN_3 (0.01% w/v) was added to 1.6 ml of MeOH. The reaction mixture was incubated at 30°C adjusting the pH with 0.25 M NaOH daily and following the reaction by TLC (AcOEt–MeOH–H_2O 8:2:0.2). After 72 h, water was evaporated and the crude residue was purified by flash chromatography to give 75 mg (0.130 mmol, 84% isolated yield) of **3a**. Amorphous solid; $[\alpha]_D$ 11.1 (*c* 0.8, MeOH); ^{1}H NMR (D_2O, 50°C): δ 7.75 (m, 5H, ArH), 5.47 (br s, 2H, CH_2Ph), 4.80 (d, 1H, J = 7.5 Hz, H-1′), 4.61 (br dd, 1H, J = 8.0, 5.0 Hz, H-2), 4.4–3.5 (m, 13H, H-5, H-6, H-7, H-8, H-9, H-10, H-2′, H-3′, H-4′, H-5′, H-6′) 2.4–2.1 (m, 3H,

H-3a, H-4), 1.81 (m, 1H, H-3b); ^{13}C NMR (75 MHz, D_2O) δ 175.8 (s, C-1), 158.8 (s, N−C=O), 137.2 (s, Ar), 129.7, 129.3, 128.5 (3s, 5 Ar C), 103.8 (d, C-1'), 80.0 (d), 79.6 (d), 79.2 (d), 76.8 (d), 76.2 (d), 74.1 (d), 73.5 (d), 71.9 (d), 69.5 (d), 68.1 (t, CH_2Ph), 61.9 (t), 61.4 (t), 55.1 (d, C-2), 53.8 (q, CH_3), 28.1 (t, 2C, C-3, C-4).

2.4. Galactosylation of p-nitrophenyl β-N-acetyl-D-glucosamine (4) in the presence of various amounts of organic cosolvents

In a total volume of 500 μl, various amounts (v/v) of organic cosolvents were added to a 50 mM Tris buffer pH 7.4 containing 15 mM **4**, 45 mM UDP-glucose, 25 mM $MnCl_2$, 0.15 U/ml GalT, 0.15 U/ml epimerase, 5 U/ml alkaline phosphatase, 0.01% w/v NaN_3 and 1 mM dithiothreitol. The reactions were incubated at 30°C and samples were removed after 3, 6, 24 and 48 h, diluted fivefold, and used for capillary electrophoresis analysis [13]. The sample solutions were hydrodinamically injected for 5 s. Separation was performed at 20 KV (anodic injection) and a current of 50 μA was generated at a temperature of 25°C. The running buffer was 0.1 M borate buffer pH 9.5. Retention times: **4**, 3.2 min; **4a**, 3.4 min.

Reactions were repeated twice and the mean values obtained are the following, reported as: *organic cosolvents, % v/v*, conversion at 3h, conversion at 6 h, conversion at 24 h and conversion at 48 h. *Blank, 0*, 14, 30, 80, 100. *Me_2SO, 5*, 8, 21, 78, 84; *Me_2SO, 10*, 5, 11, 70, 76; *Me_2SO, 15*, 3, 11, 57, 73; *Me_2SO, 20*, 1, 5, 34, 56. *MeOH, 5*, 8, 15, 69, 82; *MeOH, 10*, 3, 9, 55, 63; *MeOH, 15*, 2, 5, 36, 50; *MeOH, 20*, 1, 3, 16, 23. *EtOH, 5*, 6, 15, 80, 100; *EtOH, 10*, 4, 11, 44, 71; *EtOH, 15*, 2, 3, 31, 53; *EtOH, 20*, 1, 2, 14, 28. *Acetone, 5*, 9, 18, 43, 44; *acetone, 10*, 8, 15, 37, 41; *acetone, 15*, 4, 6, 17; 21; *acetone, 20*, 2, 4, 9, 12. *Dioxane, 5*, 9, 18, 62, n.d.; *dioxane, 10*, 5, 10, 33, n.d.; *dioxane, 15*, 4, 8, 24, n.d.; *dioxane, 20*, 3, 4, 14, n.d.. *Acetonitrile, 5*, 5, 15, 47, n.d.; *acetonitrile, 10*, 2, 6, 25, n.d.; *acetonitrile, 15*, 0, 3, 7, n.d.; *acetonitrile, 20*, 0, 0, 0, n.d.. *DMF, 5*, 5, 18, 63, 82; *DMF, 10*, 3, 12, 52, 76; *DMF, 15*, 1, 5, 35, 63. *DMF, 20*, 0, 4, 18, 30. *THF, 5*, 8, 19, 36, 36; *THF, 10*, 3, 5, 10, 11; *THF, 15*, 0, 0, 0, 0.

2.5. p-Nitrophenyl 4-O-(β-D-galactopyranosyl)-2-acetamido-2-deoxy-β-D-glucopyranoside (4a)

An 11-ml solution of 50 mM Tris buffer pH 7.4 containing **4** (80 mg, 20 mM), UDP-glucose (440 mg, 66 mM), $MnCl_2$ (25 mM), 5 U GalT, 18 U epimerase, 250 U alkaline phosphatase, dithiothreitol (1 mM) and NaN_3 (0.01% w/v) was incubated at 30°C adjusting the pH with 0.25 M NaOH and following the reaction by TLC (AcOEt–MeOH–H_2O 10:3:0.8). After 24 h, water was evaporated and the crude residue was purified by flash chromatography to give 107 mg (91% isolated yield) of **4a**. Amorphous solid; ^{1}H NMR (DMSO-d_6,): δ 8.20 and 7.18 (d, 2H each, $J = 11.0$ Hz, ArH), 5.25 (d, 1H, $J = 8.0$ Hz, H-1′), 4.40 (br d, 1H, $J = 7.5$ Hz, H-1), 1.82 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.8 (s, N−C=O), 162.3 (s, Ar), 142.1 (s, Ar), 126.0 (d, Ar), 116.9 (d, Ar), 104.1 (d, C-1′), 98.3 (d, C-1), 80.6 (d, C-4), 75.8 (d, C-5), 75.5 (d, C-5′), 73.5 (d, C-3), 72.1 (d, C-3′), 70.9 (d, C-2′), 68.4 (d, C-4′), 60.7 (t, C-6′), 59.8 (t, C.6), 23.2 (q, CH_3).

2.6. 4-O-(β-D-galactopyranosyl)-3-acetamido-2,6-anhydro-1-carbobenzyloxyamino-1,3-dideoxy-D-glycero-D-gulo-heptitol (5a)

In a total volume of 8 ml, 60 mg (0.16 mmol 20 mM) of **5** dissolved in 400 μl of Me_2SO were added to a 50 mM Tris buffer solution, pH 7.4, containing 488 mg of UDP-glucose (100 mM), 25 mM $MnCl_2$, 0.4 U/ml GalT, 1 U/ml epimerase, 7 U/ml alkaline phosphatase, 0.01% NaN_3, 1 mM dithiothreitol. The reaction was incubated at 30°C for 72 h, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 8:2:0.5). After this time, water was evaporated so as residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 8:2.5:0.5) followed by gel filtration chromatography (BIOGEL P2 resin, 2 cm i.d. × 100 cm column, water flow rate 25 ml/h) to give 65 mg (79% yield) of **5a**. Amorphous solid. ^{1}H NMR (D_2O) δ 7.52 (m, 5H, ArH), 5.21 (br s, 2H, CH_2Ph), 4.53 (d, 1H, $J = 7.8$ Hz, H-1′), 4.1–3.4 (m, 15H, H-1, H-2, H-3, H-4, H-5, H-6, H-7, H-2′, H-3′, H-4′, H-5′, H-6′), 2.12 (s, 3H, CH_3); ^{13}C NMR (75 MHz, D_2O) 175.2 (s,

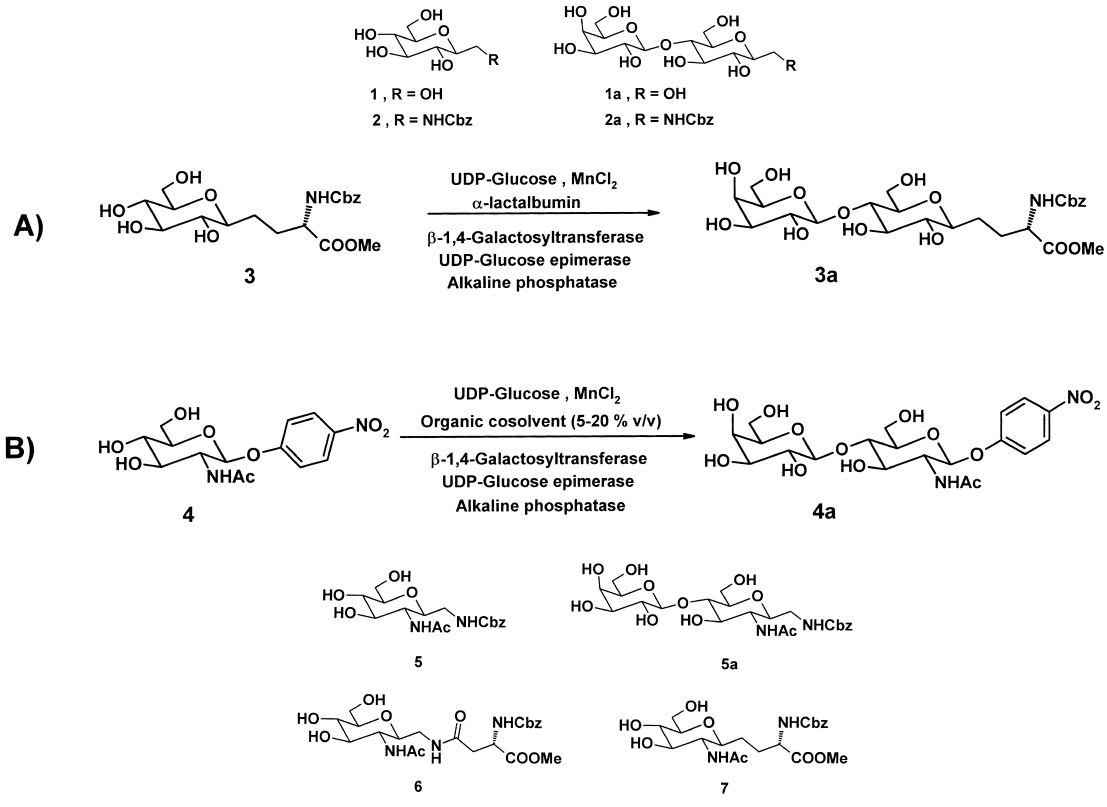
$CH_3C{=}O$) 159.1 (s, N–C=O), 137.3 (s, Ar), 129.6, 129.2, 128.5 (3s, 5 Ar C), 103.7 (d, C-1′), 79.6 (d), 79.1 (d), 77.8 (d), 76.2 (d), 74.5 (d), 73.4 (d), 71.8 (d), 69.4 (d), 67.8 (t, CH_2Ph), 61.8 (t), 61.2 (t), 60.2 (t), 53.1 (d, C-3), 42.5 (t, CH_2N), 23.0 (q, CH_3).

3. Results and discussion

The well-known multienzymatic protocol described in Scheme 1, part A [8,9] has been used for the galactosylation of the *C*-glucoside **3**. It had been previously observed that GalT is quite insensitive to the nature of the aglycone of some natural glucosides [14,15]. Accordingly, **3** proved to be an excellent substrate for this enzyme and the corresponding pseudo-lactoside **3a** was obtained in 84% isolated yield.

In nature, the most commonly observed type of glycosic bond with peptides/proteins is, by far, the β-linkage of *N*-acetyl-D-glucosamine to the amide nitrogen of asparagine side chain [2]. As a consequence, it appeared quite obvious to extend our investigation to *C*-glycosides analogues of this sugar.

As a preliminary step, the influence of organic cosolvents on GalT was investigated. Quite often, in fact, the modified sugar acceptor has a low solubility in the reaction buffer, and therefore, an adjuvant has to be added to increase the substrate concentration. On the other hand, the information that we had previously collected on the effect of cosolvents on the GalT-catalyzed galactosylation of glucose derivatives [16] could not be used, on principle, with these new sugar acceptors because of the difference of the catalytic system: the so-called "lactose synthase" (GalT associated with α-lactalbumin) glycosylates glucose and its derivatives, while GalT by itself is able to modify *N*-acetyl-D-glucosamine. As shown in Scheme 1, part B, **4** was used as a model substrate and its conversion to the galactosylated derivative **4a**



Scheme 1.

was performed in buffer alone or in the presence of different amounts (5–20% v/v) of the following water-miscible cosolvents: DMSO, MeOH, DMF, EtOH, dioxane, acetone, acetonitrile and THF.

Table 1 shows the percentage of conversion, after 24 h, in the presence of 10% or 20% v/v of these adjuvants.

The general trend that can be extrapolated from these data is not different from that previously observed [16]. The cosolvents can be roughly divided into three groups: the "good" ones — DMSO and, to a certain extent, MeOH — that can be used up to 15–20% v/v; the "bad" ones — THF and acetonitrile — that are "poisonous" for GalT even in low amounts; a third group — EtOH, DMF, dioxane, acetone — that can be used at low concentrations (the effects of these cosolvents on the UDP-glucose epimerase have been previously described [16]). To further exemplify these conclusions, the performances obtained after 48 h in the presence of three of the above-described cosolvents are depicted in Fig. 1.

Finally, we applied these findings to the enzymatic galactosylation of **5**. Due to its low solubility in the reaction buffer, the enzymatic transformation was performed in the presence of 5% v/v DMSO, which — as expected from the above-described data — did not prevent a quantitative conversion of **5** into its corresponding lactoside **5a**.

This last example shows that *N*-acetyl-D-glucosamine derivatives, modified at the anomeric center, can also be substrates for GalT. Work is in progress to synthesize the *C*-glycosides **6** and **7**, analogues of *N*-acetyl-D-glucosamine β-linked to asparagine and serine, and to submit these compounds to the catalytic action of GalT and of other glycosyltransferases.

Table 1
Galactosylation of **4** in the presence of different organic cosolvents

Cosolvent	% Relative conversion[a]	
	10% v/v	20% v/v
Blank	100	100
Dimethylsulfoxide	88	43
Methanol	69	20
Dimethylformamide	65	14
Ethanol	55	18
Acetone	46	12
Dioxane	42	17
Acetonitrile	32	0
Tetrahydrofuran	13	0

[a]Determined after 24-h reaction time by capillary electrophoresis. Conversion in the absence of cosolvents (blank) was set at 100.

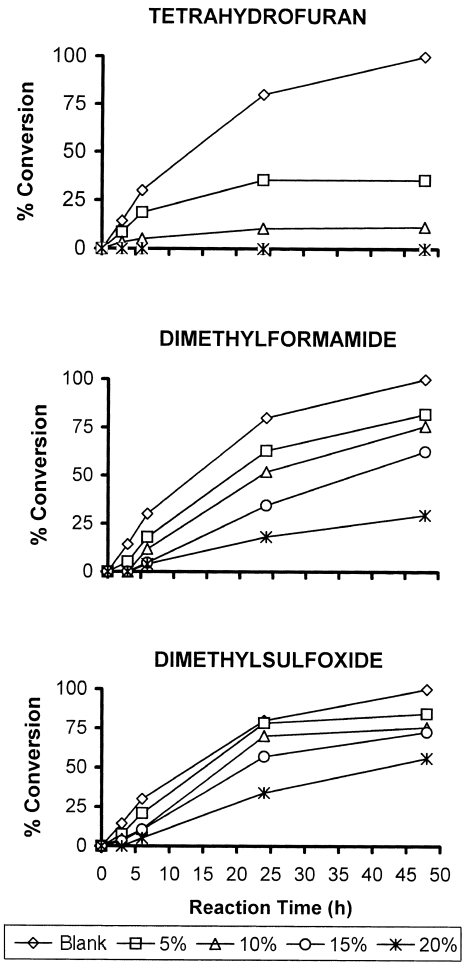


Fig. 1. Influence of various amounts of tetrahydrofuran, dimethylformamide and dimethylsulfoxide on the GalT-catalyzed galactosylation of **4**.

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