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Abstract

The synthesis of disaccharides by galactosylation of a glucose-containing cluster has been studied using β-galactosidases from different sources. Galactosylation of the cluster could be detected by ESI-MS. After size-exclusion chromatography of the reaction mixtures and subsequent cleavage of the linker, galactopyranosyl-glucose disaccharides were obtained in yield and regioselectivity dependent on the origin of the enzyme. The possibility of using this method for the synthesis of multivalent glycoconjugates is discussed.

Keywords: enzymatic synthesis; liquid-phase synthesis; glycocluster; β-galactosidase; glycosidase catalyzed transglycosylation.

1. Introduction

Major advances in the chemical synthesis of oligosaccharides have been made during the last decade due to the potential of these structures and their analogs as pharmaceutical and diagnostical reagents [1,2]. To circumvent problems associated to multiple reaction steps and the use of expensive/hazardous heavy metals salts which are use as catalysts, enzyme-

catalyzed syntheses have emerged as a valuable strategy choice [3]. Among the enzymatic methods, glycosidases have been shown to be practical for synthesis of di- and trisaccharides since they utilize readily available enzymes and substrates [4]. Besides, they can accept a broad structural range of alcohol acceptors while retaining the capacity to create stereospecifically glycosidic bonds. However, this method normally leads to complex reaction mixtures, making the purification step a difficult task. In many cases acetylation and deacetylation steps are required for the purification of the products. In order to overcome this problem, we have recently reported a new strategy based on the use of a polymer-supported sugar acceptor, which allows an easy purification of the desired products by precipitation [5]. Although the enzyme was able to recognize the sugar attached to the polymer as acceptor, yields of disaccharides were low. This was ascribed to the low loading capacity of the polymer (0.2 mmol of sugar acceptor per gram of MPEG) making difficult the use of high concentration of acceptor, as required in these glycosylations .

The aim of the present work is to evaluate the ability of these enzymes to glycosylate a multivalent glycoconjugate and the possibility of obtaining clusters with disaccharide ligands. If so, the larger molecular size of the glycocluster with respect to other byproducts would facilitate the purification by size-exclusion chromatography. In addition, the possibility of obtaining multivalent glycoclusters may be of great utility in studies of biological process where cooperative interactions between carbohydrate-biomolecule lead to an amplification of the affinity [6-8].

2. Experimental

General

β-Galactosidases and *o*-nitrophenyl β-D-galactopyranoside were purchased from Sigma. All other chemicals were from Aldrich. TLC was performed on silica-gel plates (GF_{254} Merck) with detection by charring with H_2SO_4. Column chromatography was performed on silica gel (70-230 mesh, Merck). ^1H NMR spectra were measured at 200, 300, 400 and 500 MHz. Mass spectra were recorded in a MS 1100 Hewlett Packard instrument. GC analysis was carried out on a chromatograph Hewlett Packard 5890 Series II, with FID detector, using a ultra 2 column (12 m, 0.2 mm id, and 0.33 μm film); temperature program: initial temperature 195 ^oC; rate 5 ^oC/min; final temperature 250 ^oC. Benzyl β-D-xilopyranoside was uses as internal standard. HPLC analysis was carried out on a chromatograph Waters 600 E, with UV detector (254 nm), using a reverse phase column C18 Merck Lichrospher 100 (12.5 m, 0.3 mm id, and 0.15 μm film); movil phase: (A) water containing TFA (1‰), (B) CH_3CN; gradient program: time 0 %A=90, time 2 min %A=90; time 15 min %A=60; flow 1 ml/min. Optical rotations were determined Perkin Elmer polarimeter (10 cm cells, Na-D-line; 589 nm).

4-(Hydroxymethyl)-phenylmethyl 2,3,4,6-tetra-O-*benzoyl-β-D-glucopyranoside (**5**)*

To a solution of **3** (0.5 g, 0.67 mmol) and 1,4-benzenedimethanol **(4)** (0.375 g, 2.71mmol) in CH_2Cl_2 (125 mL), TMSOTf (40 μL, 1% in CH_2Cl_2) was added, stirring at r.t. for 90 min. Then, pyridine (150 μL) was added and the reaction mixture concentrated. Purification of the residue by silica gel chromatography (Hex:AcEt, 2:1) yielded **5** (0.3 g, 64%) as a white solid: mp 61-63 ^oC; $[\alpha]^{20}_D$= +0.05 (*c* 1, $CHCl_3$); R_f 0.35 (hexanes-ethyl acetate, 1:1 v/v); ^1H NMR ($CDCl_3$, 300MHz) δ 8.14-7.86 (m, 8H, aromatic), 7.3-7.65 (m, 16H, aromatic), 5.90 (t, 1H, $J_{3,4}$=$J_{3,2}$=9.0 Hz, H-3), 5.74 (t, 1H, $J_{4,3}$=$J_{4,5}$=9.0 Hz, H-4), 5.66 (m, 1H, $J_{2,3}$=9.6 Hz, $J_{2,1}$=7.9 Hz, H-2), 4.95 (d 1H, *J*=12.0 Hz, PhC*H*), 4.9 (d, 1H, $J_{1,2}$= 7.9

Hz, H-1), 4.76 (d, 1H, *J*=12.0 Hz, PhC*H*), 4.73-4.66 (m, 3H, $J_{6a,5}$=3.3 Hz, PhCH_2, H-6a), 4.58 (dd, 1H, $J_{6a,6b}$=12.2 Hz, $J_{6b,5}$=5.3 Hz, H-6b), 4.16 (m, 1H, $J_{5,6a}$=3.3 Hz, $J_{5,6b}$=5.3 Hz, $J_{5,4}$=9.0 Hz, H-5); Anal. Calcd. for $C_{34}H_{36}O_{11}$: C, 70.38; H, 5.06. Found: C, 70.19; H, 5.01.

*Coupling of **6** with **2***

Compound **6** was prepared by treatment of **5** (120 mg, 0.17 mmol) with *1,1-carbonildiimidazol* (33 mg, 0.2 mmol) in dry dioxane (200 µL) with stirring at room temperature until the disappearance (TLC) of **5**. The reaction mixture was concentrated to give a residue (135 mg) which was added in aliquots (3 x 45 mg) to a solution of **2** [9] (20 mg, 0.028 mmol) in a mixture of nitromethane (0.875 mL) and triethylamine (0.375 mL). The mixture was heated at reflux under argon for 24 h. After concentration and purification by silica gel chromatography (toluene:ethyl acetate, 2:1 to 1:2) **7** (50 mg, 44%) was obtained as a white solid: mp 105-107 ^{o}C; $[\alpha]^{20}_{D}$= +0.1 (*c* 1, $CHCl_3$); R_f 0.23 (hexanes-ethyl acetate, 1:3 v/v); ^{1}H NMR ($CDCl_3$, 400MHz) δ 7.98-7.7 (m, 20H, Bz aromatic), 7.14-7.48 (m, 100H, Bn aromatic), 5.78 (t, 5H, $J_{3',2'}$=$J_{3',4'}$=9.6 Hz, 5H-3'), 5.61 (t, 5H, $J_{4',3'}$=$J_{4',5'}$=9.6 Hz, 5H-4'), 5.52 (t, 5H, $J_{2',1'}$= 8 Hz, $J_{2',3'}$=9.6 Hz, 5H-2'), 5.3 (bs, 5H, 5N*H*CO), 5.0-4.4 (m, 36H, H-1, 5H-6a', 5H-6b', 5H-1', 10PhCH_2), 4.04 (m, 5H, 5H-5'), 3.7-3.1 (m, 26H, 5C$H_2$$CH_2NH_2$, 5$CH_2CH_2$$NH_2$, H-2, H-4, H-3, H-5, H-6a, H-6b); ^{13} C NMR ($CDCl_3$, 50 MHz) δ 165.9, 165.6, 165.0, 164.8, 156.2, 136.4, 136.0, 133.2, 133.0, 129.6, 129.5, 129.1, 128.7, 128.2, 128.1, 127.7, 99.4, 72.8, 72.1, 71.7, 70.1, 69.6, 66.1, 62.9; Anal. Calcd. for $C_{231}H_{207}N_5O_{66}$: C, 67.52; H, 5.08; N, 1.71. Found: C, 67.23; H, 5.15; N, 1.39.

*Glycocluster **1***

A solution of **2** (0.24 g, 0.058 mmol) in dry MeOH (7 mL) and THF (1mL) was treated with NaOMe (1 mL, 0.5 M in MeOH) at room temperature for 0.5 h. Then, water (2 mL) was added and the solution was neutralized with Amberlite IR 120 (H^+). The mixture was filtered and the filtrate concentrated. The residue was dissolved in water (109 ml) and the solution extracted with ethyl ether (5 x 10 mL). Lyophilization of the water solution gave **1** (0.12 g, 99%) as a white solid: mp 111-114 ^oC; $[\alpha]^{20}_D$= -0.03 (*c* 0.6, CH_3CN-H_2O, 9:1 v/v); R_f 0.24 (isopropanol-NH_3-H_2O, 7.5:0.5:2.5 v/v); ESI-MS ($[M + 2Cl^-]^{2-}/2$) = 1048.0 m/z; ^1H NMR (D_2O-CD_3CN, 2:1 v/v, 500MHz) δ 5.48 (m, 10H, 5OCOC*H*$_2$Ar), 5.32 (m, 6H, 5OC*H*HAr, H-1), 5.1 (m, 5H, 5OCH*H*Ar), 4.86 (d, 5H, $J_{1',2'}$= 8 Hz, H-1'), 4.28 (dd, 5H, $J_{6a',6b'}$= 12 Hz, 5H-6a'), 4.2-3.6 (m, 51H, 5H-6b', H-6b, H-6a, H-5, H-4, H-3, H-2, 5C*H*$_2$$CH_2$NH, 5$CH_2$C*H*$_2$NH, 5H-2', 5H-3', 5H-4', 5H-5'); ^1H-^{13}C HSQC: δ 5.34 (H-1), 4.84 (H-1'), 4.28 (H-6a'), 4.15 (OC*H*$_2$$CH_2$), 4.1 (H-6b'), 4.1 (H-6a), 3.98 (H-3), 3.96 (H-5), 3.9 (H-6b), 3.82 (H-2'), 3.76 (H-5'), 3.76 (H-3'), 3.72 (H-2), 3.7 (H-4'), 3.68 (H-4).

*Solubity of **1** in different water-organic solvent mixtures*

10 mM of **1** was dissolved in water or water containing 10% or 20% of DMF, THF, DMSO, acetone, dioxane or DGDEE. The mixtures were stirred at room temperature for 5 min and, then, centrifuged for 1 min. 10 μl of the supernatant were dissolved in 90 μl of H_2O:CH_3CN (1:1, v/v) to determine the concentration of cluster by HPLC.

Activity and stability of β-galactosidases in different water-organic solvent mixtures

Stability of β-galctosidases from *E. coli, A. oryzae* and bovine liver was measured as the half-life of the enzymes at 37 ˚C in presence of 10% or 20% of DMF, THF, DMSO, acetone, dioxane or DGDEE. 120 U/ml of the enzyme (25 μl) was incubated at 37 ^oC in buffer (175 μl of K_2HPO_4 50 mM buffer, pH=7.0 containing, $MgCl_2$ 1 mM, β-

mercaptoethanol 5 mM, for the enzyme from *E. coli* and K_2PO_4 50 mM, pH=7.3 for the enzymes from *A. oryzae* and bovine liver) in the presence or in the absencee of organic solvent. The residual activity was evaluated as follows: at different times, aliquots of 5 μl were taken off and added to 50 mM solution of -β-D-galactopyranoside in buffer (95 μl), and the mixture was incubated at 37 ^oC for 30 min. After quenching by addition of 700 μl Na_2CO_3 (1M), the enzymatic activity was measured by the absorbance at 420 nm of the *o*-nitrophenol released.

The activity of the β-galactosidases in solutions containing the organic solvent, was measured by the same procedure.

*Enzymatic tranglycosilation of **1***

Using β-galactosidase from *E. coli*: A solution of **1** (10 mM) and *o*-nitrophenyl β-D-galactopyranoside (**8**, 25 mM) in a mixture of buffer (225 μl, K_2HPO_4/KH_2PO_4 50 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 5 mM, pH=7.0) and dioxane (25 μl) was incubated in the presence of β−galactosidase from *E. coli* (2 units) at 37 ^oC. Three additional aliquots of **8** were added during the reaction. After 8 h the incubation was stopped by heating at 100 ^oC during 10 min. The mixture was concentrated and the residue fractionated on a Biogel P2 column (water/acetonitrile 4:1). The first eluted fraction (1mg) was dissolved in a mixture of methanol (125 μl), water (125 μl), and acetic acid (10 μl), and the solution was stirred under hydrogen atmosphere in the presence of Pd/C for 24 h. The catalyst was filtered out and the filtrate was analysed by gas chromatography. An aliquot (100 μl) was concentrated and the residue treated with pyridine (5 μl) containing 1 mM of benzyl xylopyranoside as reference, trimethylsilylimidazole (5 μl) heating at 60 ^oC for 30 minutes. The silylated sugars were analyzed by GC. The retention times were compared to those of known

sugars. The amount of disaccharides formed were determined from previous calibration curves obtained for each sugar.

Using β-galactosidase from *A. Oryzae*: The incubation was carried out under similar conditions as described above using the β-galactosidase from *A. Oryzae* (2 units) in buffer (K_2HPO_4/KH_2PO_4 50 mM, pH=5.5) at 37 ºC for 6 h.

Using β-galactosidase from bovine liver: The incubation was carried out under similar conditions as described above using the β-galactosidase from bovine liver (2 units) in buffer (K_2HPO_4/KH_2PO_4 50 mM, pH=7.3) at 37 ºC for 6 h.

*Enzymatic tranglycosilation of **9***

To a solution of **9** (50 mM) and *o*-nitrophenyl-β-D-galactopyranoside (50 mM) in a mixture of buffer (333 μl, K_2HPO_4/KH_2PO_4 50 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 5 mM, pH=7.0) and dioxane (37 μl) was incubated in the presence of β–galactosidase from *E. coli* (2 units) at 37 ºC. Three additional aliquots of **8** were added during the reaction. After 4 h the incubation was stopped by heating at 100 ºC during 10 min. The reaction mixture was concentrated and the residue dissolved in a mixture of methanol (125 μl), water (125 μl), and acetic acid (10 μl), and the solution was stirred under hydrogen atmosphere in the presence of Pd/C for 24 h. The catalyst was filtered out and the filtrate was analysed by gas chromatography as described above.

A similar procedure was followed using the enzymes from *A. Oryzae* (2 units) and bovine liver (2 units) in buffer (K_2HPO_4/KH_2PO_4 50 mM, at pH's=5.5 and 7.3, respectively).

3. Results and discussion

Based on a recent work [9] describing the synthesis of the carbohydrate derived pentamine **2**, the glycocluster **1**, in which the amine groups are attached to terminal glucosides using a

residue of 1,4-benzenedimethanol as linker, was designed and synthesized (Scheme I). This linker is bound to the anomeric carbon of the sugar, forming a glycosidic bond, and to the amine groups through a carbonyl group, forming a carbamate. In this manner, the glucosides can be released from the cluster either by hydrogenolysis of the benzylic bonds or by acid hydrolysis of the carbamates.

The synthesis of **1** is outlined in Scheme II. Glycosylation of the tetra-*O*-benzoyl-glucopyranosyl trichloroacetimidate (**3**) [10] with 1,4-benzenedimethanol (**4**) in the presence of TMSOTf afforded the β-glucopyranoside **5** in 64% yield. Compound **5** was treated with carbonyl-diimidazol to give the imidazolyl carbonate **6** which was immediately coupled with the pentamine **2** [9] to give the protected glycocluster **7** in 44% yield. The ^{1}H-NMR spectrum of **7** was in agreement with the presence of five benzoylated β-glucopyranosyl residues. Finally, debenzoylation of **7** using NaOMe furnished compound **1**, which was fully caractherized by NMR and MS.

Glycocluster **1** was soluble in buffer at only 1.25 mM concentration, which is low for the usually required concentrations in glycosidase-catalyzed reactions. We therefore tested the solubility of **1**, expressed in mM concentration in table 1, using different water-miscible organic solvents. The best results are obtained in 10-20% dioxane. In order to know the effect of the cosolvents on the enzyme activity, we then checked the stability and catalytic activity of the β-galactosidase from *E. Coli* in the solvent mixtures (table 1 entries 2 and 3). Taking together the results of the different experiments, the reaction medium of choice was 10% dioxane, since the stability and catalytic activity of the enzyme is almost as high as in buffer while the solubility is five-fold increased. The β-galactosidases from *Aspergillus oryzae* and bovine liver showed a similar behavior in this solvent mixture (table 2). Therefore, the β-galactosidase-catalyzed glycosylations of **1** were carried out in 10% dioxane.

Glycosylations were perfomed by incubation of **1** (10 mM) and *o*-nitrophenyl β-D-galactopyranoside **8** (100 mM), as glycosyl donor, in the presence of the β-galactosidase at 37 ºC. The progress of the reaction was monitored by tlc. The procedure to isolate and identify the disaccharides formed from the galactosylation of **1** is outlined in Scheme III. When all substrate donor was consumed, the reaction was stopped by heating at 100 ºC, concentrated and fractionated on a size exclusion chromatographic column (Biogel P2). The difference in size between glycocluster and other byproducts allowed a rapid and efficient separation. Thus, the first eluted fraction contained **1** and its glycosylated derivatives, as indicated by their MS (Figure 1). This fraction was submitted to hydrogenolysis to release disaccharides and unreacted glucose, the ratio of which was determined by GC (Figure 2). It is worth noting that in this fraction no other by-product (that is, galactose, unreacted donor, galactosyl-galactose and *o*-nitrophenyl galactosyl-galactopyranoside disaccharides) was present.

Table 3 summarizes the results of the transglycosylations using β-galactosidases from *Escherichia coli*, *Aspergillus orizae* and bovine liver. The three enzymes are able to catalyze the glycosylation of the cluster. Thus, galactosil-glucose disaccharides were obtained after cleavage of the linker. Yield and regioselectivity of disaccharides were dependent on the origin of the enzyme used. While the β-galactosidase from *E. coli* gave Gal(1-3)Glc disaccharide as major product, the enzyme from bovine liver afforded mainly the (1-6) regioisomer. The enzyme from *A. Oryzae* gave, however, poor yield and regioselectivity of Gal-Glc disaccharides.

Previous works [11,12] on glycosidase-catalyzed synthesis of disaccharides have shown that the nature of the aglycon structure in the monosaccharide acceptor can significantly alter the result of the glycosylations. Regarding this point, it seemed interested to compare the results obtained with the cluster with those of glucopyranoside **9**,

which contains a benzyl group as aglycon. Compound **9** is a good model of the terminal fragment of the cluster arms. The glycosylations of **9** with the three enzymes were carried out under the same conditions; the results are shown in Table 4. The reactions with the β-galactosidases from *E. coli* and bovine liver gave disaccharides in yield and regioselectivity similar to those obtained with cluster **1**. This may indicate that in the cluster there is little influence of the core and the adjacent arms on the galactosylation of the different benzyl glucopyranoside moieties. On the other hand, the reaction of **9** in the presence of the enzyme from *A. Oryzae* led to a significant change of the regioselectivity, giving in this case the (1-6) disaccharide as the only regioisomer, although in low yield.

3. Conclusions

In this preliminary work we showed that glycosidases are able to catalyze the glycosylation of a glycocluster with the regioselectivity modulated by the origin of the enzyme. A size-exclusion column chromatography of the reaction mixture allowed the purification of the cluster efficiently, which, after cleavage of the linker, afforded only desired disaccharides and unreacted acceptor. One still can envisage an improved system using glycoclusters of larger molecular size that can be separated from the reaction mixtures by a simple ultrafiltration. For the possibility of using the glycosylated clusters as multivalent glycoconjugates in biological studies, yields of glycosylations must be improved. In this sense, the synthesis of glycocluster acceptors with increased water solubility would allow to carry out glycosylations at higher substrate concentration and, therefore, to obtain better yields of disaccharides.

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Table 1.

Study of the effect of different co-solvents on glycocluster **1** solubility and on *E. coli* β-galactosidase activity.

	Co-solvent (%)												
	—	Acetone		DGDEE		Dioxane		DMF		DMSO		THF	
		10	20	10	20	10	20	10	20	10	20	10	20
Glycocluster Solubility (mM)	1.25	2.02	3.65	2.42	3.6	10	10	2.9	4.4	2.36	2.46	2.07	3.9
E. coli β-galactosidase stability[a] ($t_{1/2}$ h)	>24	N.A	1.5	N.A	0.05	>24	0.3	N.A	0.5	>24	N.A	N.A	0.05
E. coli β-galactosidase activity[b] (%)	100	N.A	54	N.A	2	87	37	N.A	44.5	100	N.A	N.A	5

[a] The stability of the enzyme is expressed as the time (h), at wich the activity dropped to 50% ($t_{1/2}$).
[b] The activity is referred to the one shown in absence of co-solvent.
N.A = Not assayed.

Table 2.

Stability and activity of the β-galactosidase from A. oryzae and bovine liver, in 10% of dioxane.[a]

	A. oryzae β-galactosidase	Bovine liver β-galactosidase
Stability[a] ($t_{1/2}$ h)	>24	>24
Activity[b] (%)	62.5	68

[a] The stability of the enzyme is expressed as the time (h), at wich the activity dropped to 50% ($t_{1/2}$).
[b] The activity is referred to the one shown in absence of co-solvent.

Table 3.

Transglycosidation of **1** catalyzed by β-galactosidases from different sources.

β-Galactosidase	Yield (%)	Regioselectivity (%)[a]		
		Gal(1—3)Glc	Gal(1—4)Glc	Gal(1—6)Glc
E. coli	25	66	8	26
A. oryzae	5	32	16	52
Bovine liver	17	7	2	91

[a] Regioselectivity is expressed as the percentage of each regioisomer with respect to the total amount of disaccharide.

Table 4.

Transglycosidation of **9**, catalyzed by β-galactosidase from different sources.

β-Galactosidase	Yield (%)	Regioselectivity (%)[a]		
		Gal(1—3)Glc	Gal(1—4)Glc	Gal(1—6)Glc
E. coli	32	68	N.D	32
A. oryzae	7	N.D	N.D	100
Bovine liver	14	N.D	N.D	100

[a] Regioselectivity is expressed as the percentage of each regioisomer with respect to the total amount of disaccharide.
N.D = Not detected.

Legend of the Figures

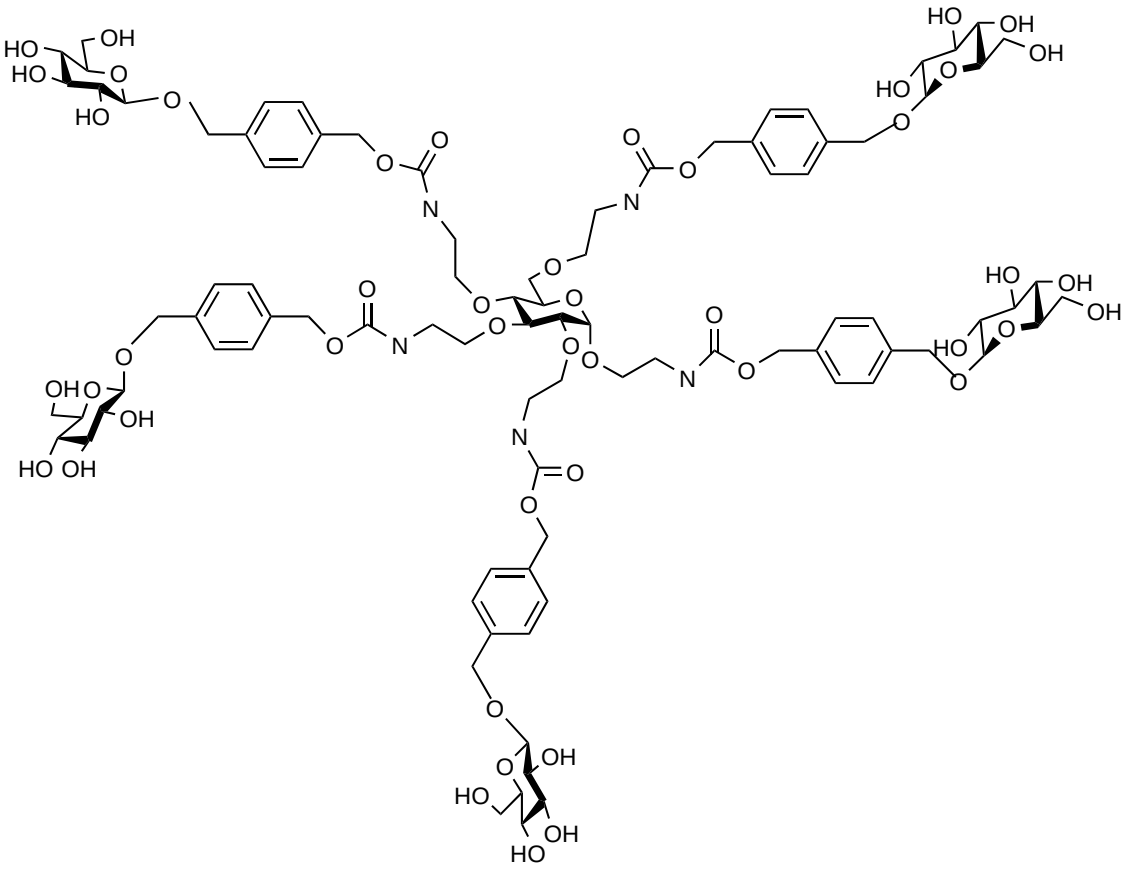
Scheme I.- Structure of glycocluster **1** and of the carbohydrate derived pentamine **2**.

Scheme II.- Synthesis of glycocluster **1**.

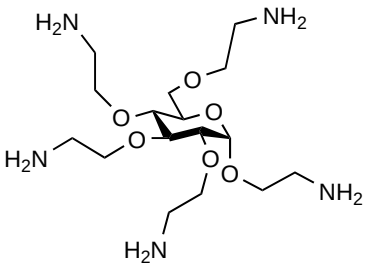
Scheme III.- Procedure to isolate and identify the disaccharides formed from the galactosylation of **1**. Step 1: β-Galactosidase catalyzed transglycosilation of **1**; step 2: Isolation of the glycocluster containing mono and disaccharides, by gel filtration; step 3. Release of disaccharides and unreacted glucose by hydrogenolysis.

Figure 1.- ESI-mass spectrum of the fraction contained **1** and its glycosylated derivatives. The peaks corresponding to glycocluster **1** (($[M + 2Cl^-]^{2-}/2$) = 1048.0 m/z and $[M + Cl^-]^{2-}$ = 2062.0 m/z); glycoclustur containing one galactose residue(($[M + 2Cl^-]^{2-}/2$) = 1130.1 m/z and $[M + 2Cl^-]^{2-}$ = 2224.2 m/z) and glycocluster containing two galactose residues (($[M + 2Cl^-]^{2-}/2$) = 1210.9 m/z) were present.

Figure 2.- GC analysis of the fraction containing **1** and its glycosylated derivatives after hydrogenolysis. I.S= benzyl β-D-xylopyranoside.

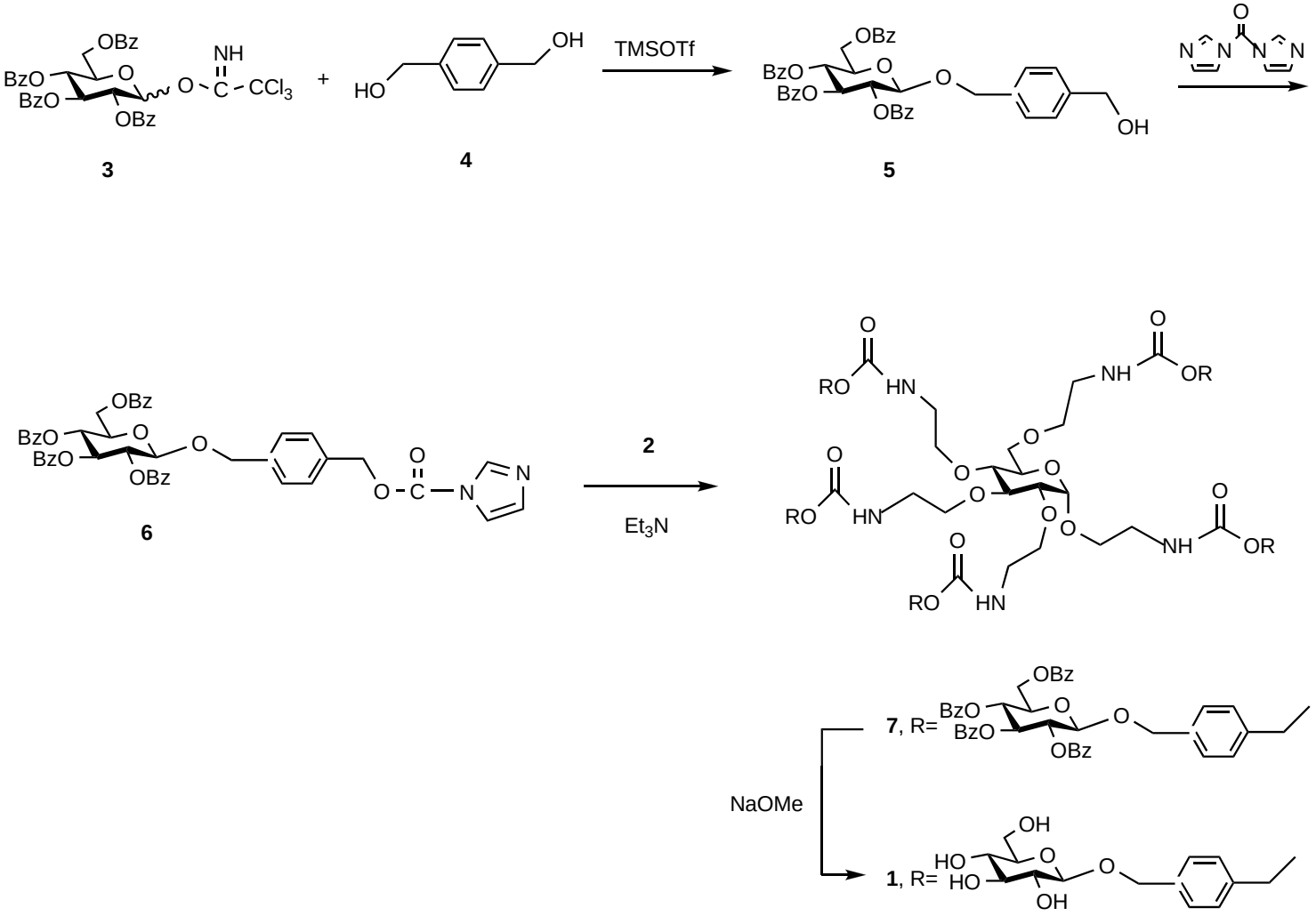


1

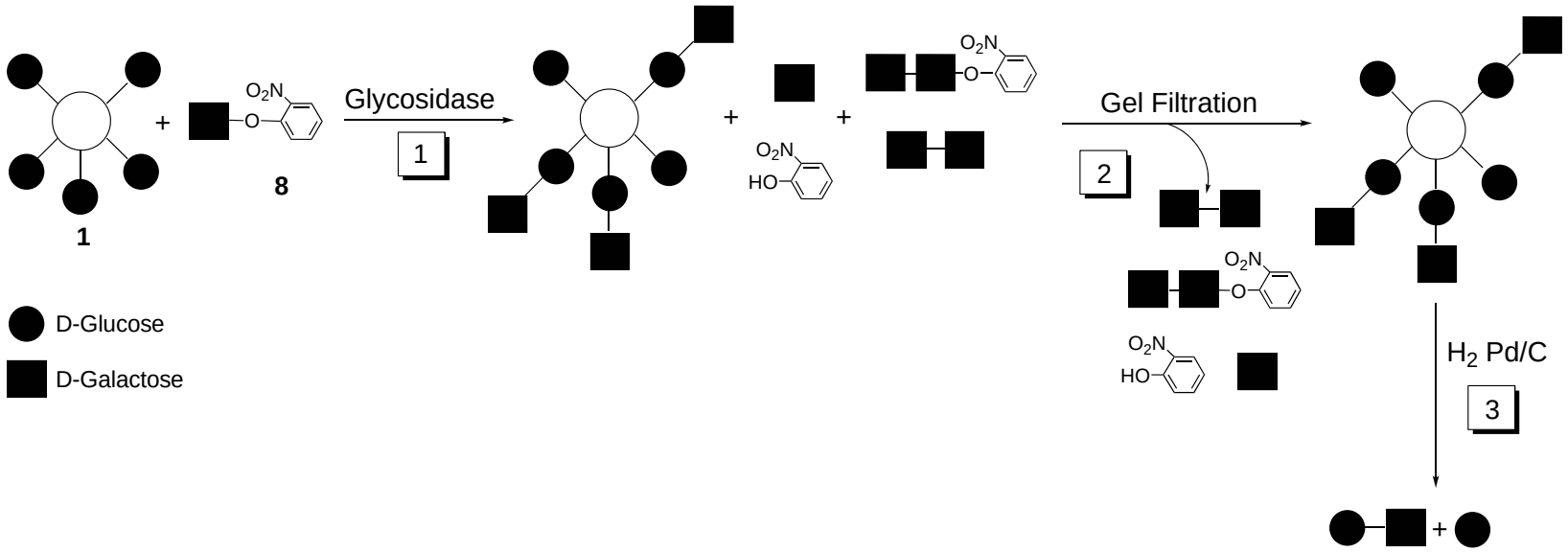


2

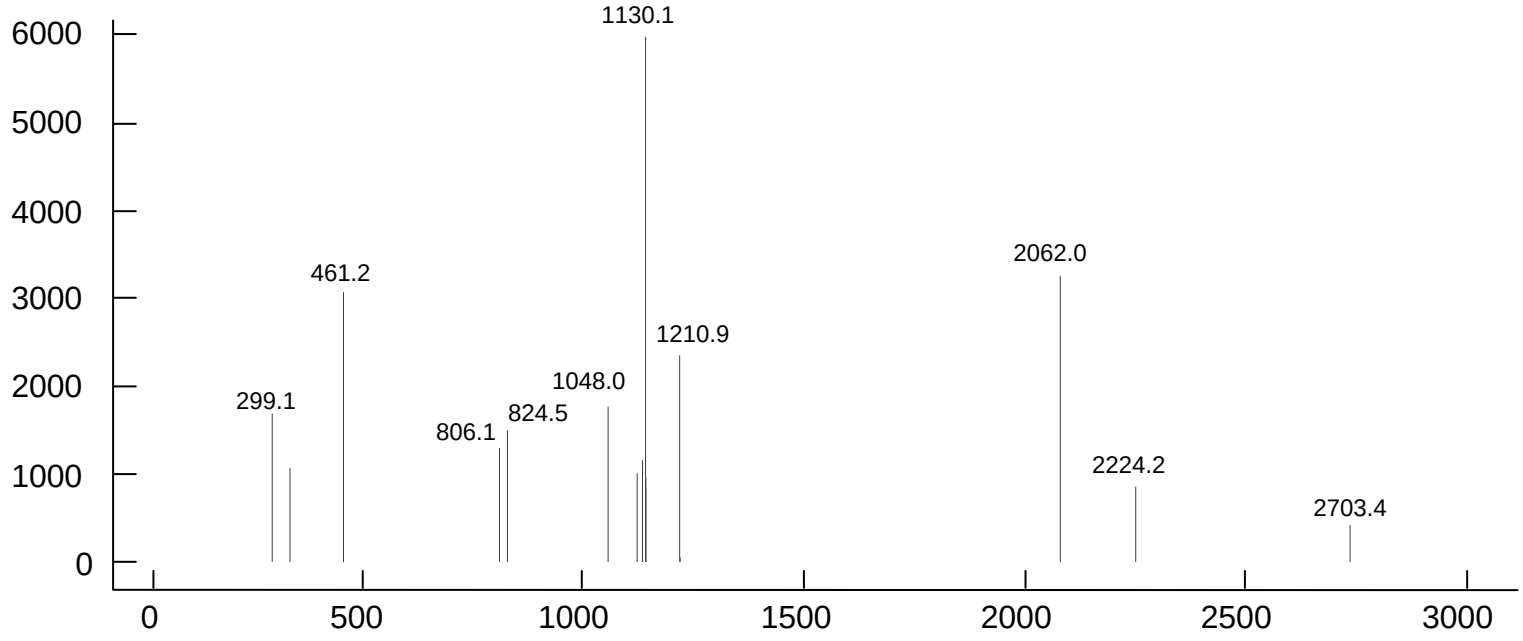
Scheme I

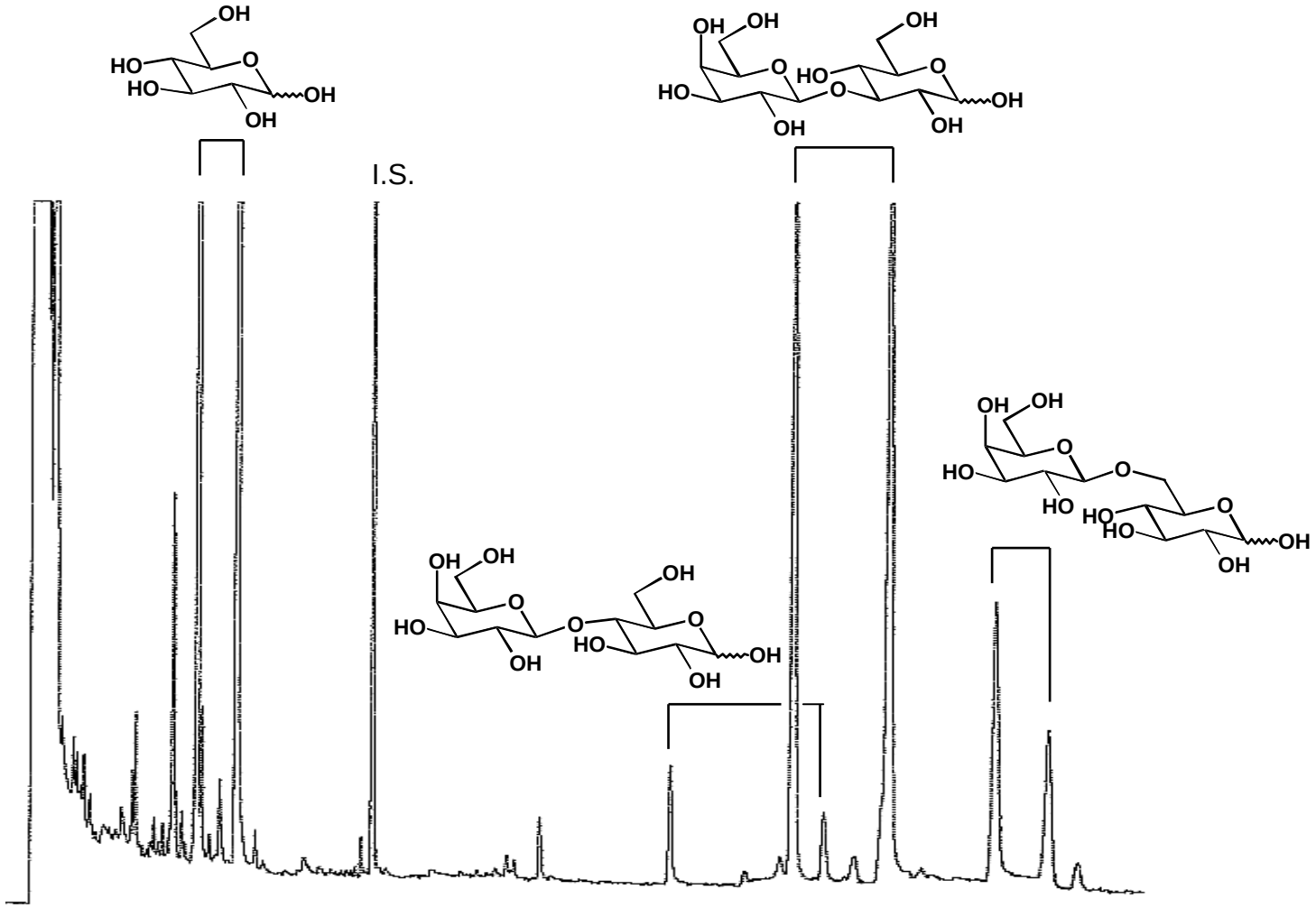

OBz
BzO
BzO
OBz
NH
O
C
CCl3
3
+
HO
OH
4
TMSOTf
5
O
N
N
N
N
6
2
Et3N
RO
HN
NH
OR
7, R=
1, R=
NaOMe
HO

Scheme II


1
8
Glycosidase
1
Gel Filtration
2
H2 Pd/C
3
D-Glucose
D-Galactose

Scheme III


6000
5000
4000
3000
2000
1000
0
0
500
1000
1500
2000
2500
3000
1130.1
461.2
2062.0
1210.9
1048.0
299.1
824.5
806.1
2224.2
2703.4


I.S.