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Oxidation of galactose and derivatives catalysed by galactose oxidase: structure and complete assignments of the NMR spectra of the main product

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Abstract

This paper deals with the analysis of the structure of 6-oxogalactose and 6-oxogalactosides obtained from the oxidation reaction catalysed by galactose oxidase. A complete analysis of the ^{1}H and ^{13}C NMR spectra showed that 6-oxogalactose and 6-oxogalactosides were mainly present as the hydrate of the monomeric form.

Keywords: Galactose oxidase; Hydrates of 6-oxogalactosides; NMR parameters

1. Introduction

Galactose oxidase (GO, EC 1.1.3.9) is known to convert selectively the CH_2OH group of galactose and galactosides into an aldehyde function [1–5]. This reaction has also been applied to polysaccharides containing galactosyl units and the oxidized polysaccharide has been used as a cross linker reagent [6]. Although the regioselectivity of GO is undisputed, the structure of the oxidized products has been a subject of discussion. Thus, in aqueous solution, the free aldehyde represents less than 1% while the hydrate form seems to be the most abundant one, at least in the case of galactosides [4,7]. Conversely, a dimeric structure was also found in the case of methyl β-6-oxogalactoside [7]. In a recent study of galactose dialdehyde, Kieboom and co-workers [8] have described the presence of a cyclic hemiacetalic form as a predominant structure. In organic solvent, an increase in the concentration of free aldehyde was observed although this later was rapidly transformed into many unidentified compounds [8]. Being interested in the glycosylation of 6-amino-6-deoxycyclodextrins by means of the amino-reduction reaction in the presence of 6-oxogalactosides [9,10], we needed to know the structure of the predominant form in water solution of 6-oxogalactose and of 6-oxogalactosides. Thus, we have undertaken a systematic NMR study of these compounds in order to get more information on that topic. The complete assignment of the ^{1}H and ^{13}C NMR spectra of the main structure obtained in the GO-catalysed oxidation has shown that, in aqueous media, the hydrate form is predominant

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for 6-oxogalactose and for all 6-oxogalactosides studied.

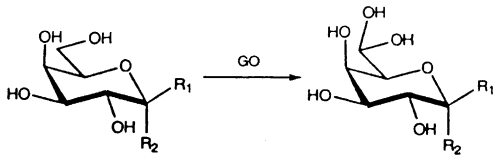
2. Results and discussion

The reaction catalysed by galactose oxidase (GO) and the compounds **1**–**6** studied in this work are described in Table 1. This enzyme uses the prosthetic cofactor FAD/$FADH_2$. Since the enzymatic activity is inhibited by an excess of H_2O_2, it is necessary to introduce a recycling step by means of catalase, which allows the reduction of the peroxide with the formation of water and dioxygen (see Section 3).

The structure of compounds **1**–**6** was determined by means of NMR spectroscopy (^{1}H NMR spectrum of **2** is given in Fig. 1). The NMR parameters of the most abundant species are listed in Table 2 (proton chemical shifts), Table 3 (proton–proton coupling constants), and Table 4 (carbon chemical shifts). The ^{1}H NMR chemical shifts of the corresponding galactosides are also included in Table 2 for comparison.

Table 1
List of the hydrates of 6-oxogalactosides studied

Substrate	Main product	R^1	R^2
α-D-Gal	**1**α	H	OH
β-D-Gal	**1**β	OH	H
Me α-D-Gal	**2**	H	O-Me
*p*NP α-D-Gal	**3**	H	O-*p*NP
*p*NP β-D-Gal	**4**	O-*p*NP	H
α-D-Mel	**5**α	H	6-*O*-α-Gluc
β-D-Mel	**5**β	H	6-*O*-β-Gluc
α-D-Lac	**6**α	4-*O*-α-Gluc	H
β-D-Lac	**6**β	4-*O*-β-Gluc	H



The complete analysis of the NMR spectra of compounds **1**–**6** was achieved with the help of well-known one- and two-dimensional (2D) NMR experiments (proton–proton, carbon–proton correlations and HO-

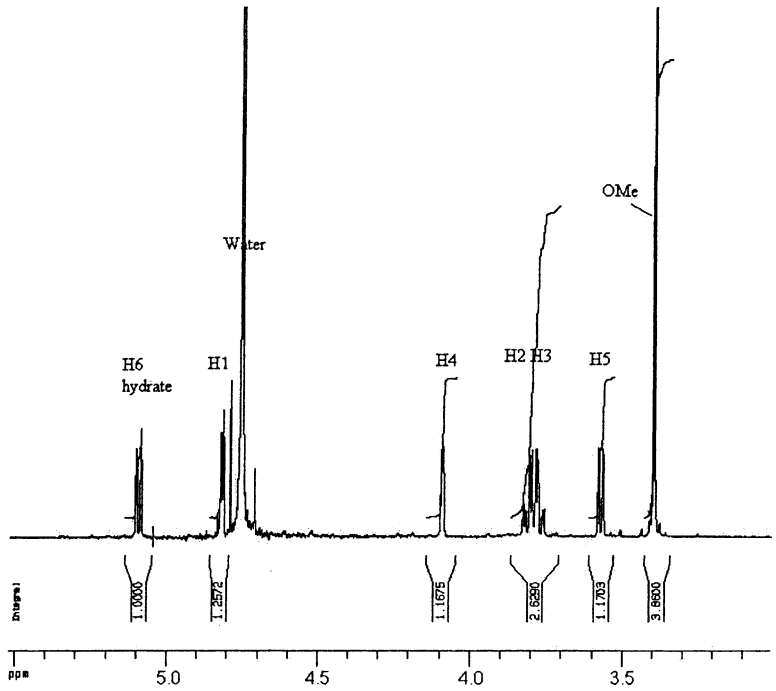


Fig. 1. NMR spectrum of the hydrate of methyl α-D-*galacto*-hexodialdo-1,5-pyranoside **2** in D_2O at 500 MHz (TSP, internal reference).

Table 2
^{1}H Chemical shifts (ppm) of hydrates of 6-oxogalactosides **1**–**6** (solvent D_2O, methyls of TSP as an internal reference)

	H-1	H-2	H-3	H-4	H-5	H-6A, H6B
1						
α	5.26	3.8	3.83	4.12	3.79	5.09
β	4.57	3.49	3.62	4.06	3.40	5.12
Gal[a]						
α	5.25	3.84	3.79	3.97	4.07	3.66–3.75
β	4.57	3.48	3.63	3.92	3.70	3.66–3.75
2	4.83	3.81	3.77	4.10	3.57	5.11
3	5.84	4.05	4.09	4.19	3.66	5.08
*p*NP α-Gal[a]	5.84	4.05	4.12	4.07	3.97	3.70, 3.73
4	5.10	3.88	3.79	4.09	3.64	5.05
5						
I						
α	5.24	3.54	3.69	3.49	4.01	3.72, 4.04
β	4.67	3.26	3.5	3.51	3.64	3.78, 3.97
II	4.99	3.84	3.89	4.14	3.69	5.11
Mel[a]						
I						
α	5.24	3.55	3.72	3.51	4.01	3.71, 4.01
β	4.68	3.26	3.51	3.53	3.65	3.78, 3.96
II	4.99	3.83	3.90	4.01	3.97	3.75
6						
I						
α	5.22	3.58	3.84	3.66	3.93	3.86, 3.88
β	4.67	3.29	3.67–3.63	3.67–3.63	3.61	3.80, 3.95
II	4.47	3.58	3.67	4.09	3.47	5.16
Lac[a]						
I						
α	5.22	3.59	3.83	3.65	3.95	3.85, 3.90
β	4.66	3.28	3.63–3.68	3.65	3.60	3.79, 3.94
II	4.44	3.59	3.65	3.92	3.73	3.76, 3.81

2: δ(OMe) = 3.23 ppm; **3**: δ(H aromatic) = 7.29 and 8.25 ppm; **4**: δ(H aromatic) = 7.16 and 8.19 ppm.
[a] NMR chemical shifts of galactosides from literature data [12–14]) are also given for comparison.

HAHA sequence for disaccharides). The analysis of the spectra showed the following features:

- A resonance around 5.1–5.2 ppm, present for all compounds in the ^{1}H NMR spectra, had formerly been assigned to the hydrate form of methyl β-6-oxogalactoside [7]. Other absorptions observed in the range 4.6–5.3 ppm must be attributed to anomeric protons for the following reasons: in each case the chemical shifts were similar to those of the corresponding galactoside (see Table 2) and the coupling constants were in accordance with $^4C_1$ pyranose forms (see Table 3, for example H-2(II) of 6-oxolactose (**6**) at 3.58 ppm has two large coupling constants, $J_{1,2} = 7.5$ Hz and $J_{2,3} = 10.0$ Hz).
- In the conditions used to perform the enzymatic oxidation and to record the NMR spectra, dimeric structures like that observed by Maradufu and Perlin (see Scheme 1) [7] can be excluded. For instance, the ^{13}C spectrum of compound **2** was composed of only seven resonances indicating a monomeric structure. Such dimeric forms are probably present, but in very low amounts in equilibrium with the hydrates. Since Marafudu and Perlin observed their dimer after the acetylation of the oxidation product, it is likely that this reaction induced modifications of

Table 3
^1H–^1H coupling constants (Hz) of hydrates of 6-oxogalactosides (**1**–**6**) (solvent D_2O)

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
1							
α	3.5	10.5	3.0	0.9	7.3		
β	7.8	9.8	3.4	1.2	7.2		
2	3.6	10.4	3.1	0.9	7.4		
3	3.4	10.7	3.2	1.0	7.3		
4	7.6	9.9	3.5	1.0	7.3		
5							
I							
α	3.8	9.9	nd	nd	nd	4.9	−11.3
β	7.9	9.1	nd	nd	2.0	4.9	−11.3
II	3.6	10.5	3.1	0.9	7.3		
6							
I							
α	3.3	9.1	8.0	2.6	2.1	4.5	−12.3
β	7.4	8.3	nd	nd	2.4	4.7	−12.4
II	7.5	10	3.4	1.0	7.2		

nd: not determined.

the concentrations of the different species in equilibrium. Furthermore, the stability of the peracetylated 6-oxogalactosides is lower than that of the native one since, for instance, β-4,5-elimination is greatly favoured. Another possible dimer would be the symmetrical one resulting from the hemiacetalisation between the C-6 of an aldehyde molecule and the 6′-OH of the hydrate of another one (see Scheme 1). The NMR spectra of such a dimer would present similar multiplicities to that of the monomeric hydrate. In order to discriminate between the two possibilities, we have determined the molecular mass by means of the ^1H NMR spectra of known concentrations of the hydrates **1**–**6** in the presence of known amounts of tetramethylurea ($\delta = 2.41$ ppm) used as a reference. In each case, our results indicate the presence of a monomeric compound. In accordance with previous work on that topic [7,8], we have also noted the presence of very low amounts of the free aldehydic group. A very small peak around 9.5 ppm due to the resonance of the CHO was usually observed. The same was true for ^{13}C spectra (carbonyl carbon at 204 ppm).

The oxidation of D-galactose is of great interest since it produces a racemic mixture of D- and L-β and α-*galacto*-hexodialdo-1,5-pyranoses, thus opening an entry to L-saccharides (see Scheme 2).

Table 4
^{13}C chemical shifts (ppm) of hydrates of 6-oxogalactosides (Solvent D_2O, methyls of TSP as an internal reference)

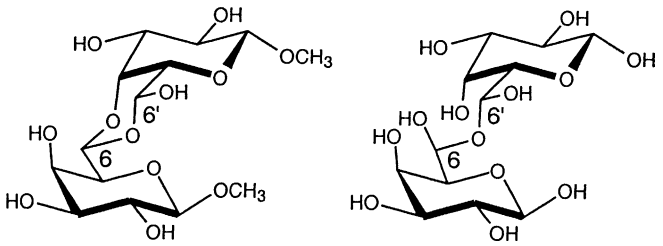
	C-1	C-2	C-3	C-4	C-5	C-6
1						
α	95.0	70.9	71.9	71.5	74.9	91.2
β	99.3	74.4	75.5	71.0	79.6	90.9
Gal[a]						
α	94.9	71.0	71.8	71.9	73.1	63.8
β	99.1	74.5	75.5	71.4	77.8	63.6
2	102.2	70.8	72.2	71.4	75.2	91.2
3	99.7	70.6	72.1	71.4	76.7	91.1
*p*NP α-Gal[a]	99.7	70.6	72.1	71.8	74.9	63.7
4	102.5	74.7	72.5	70.4	79.6	90.4
5						
I						
α	94.9	74.1	75.4	72.4	72.9	68.8
β	98.8	76.8	78.6	72.1	77.2	68.8
II	101.0	71.1	72.2	71.4	75.5	91.3
Mel[a]						
I						
α	94.9	74.3	75.8	72.4	72.9	68.8
β	98.9	76.9	78.7	72.3	77.2	68.7
II	101.0	71.3	72.3	72.0	73.7	63.9
6						
I						
α	94.5	73.4	74.2	81.2	71.3	62.8
β	98.5	76.4	75.3	81.1	77.6	62.9
II	105.9	73.7	77.1	70.7	79.6	90.7
Lac[a]						
I						
α	94.5	72.8	73.8	81.2	74.1	62.7
β	98.4	76.5	77.0	81.1	77.5	62.8
II	105.6	73.6	75.2	71.3	78.0	63.7

2: δ(OMe) = 57.9 ppm; **3**: δ(aromatic) = 119.7, 128.8, 145.2 and 164.4 ppm; **4**: δ(aromatic) = 118.8, 128.4, 144.9 and 164.2 ppm.

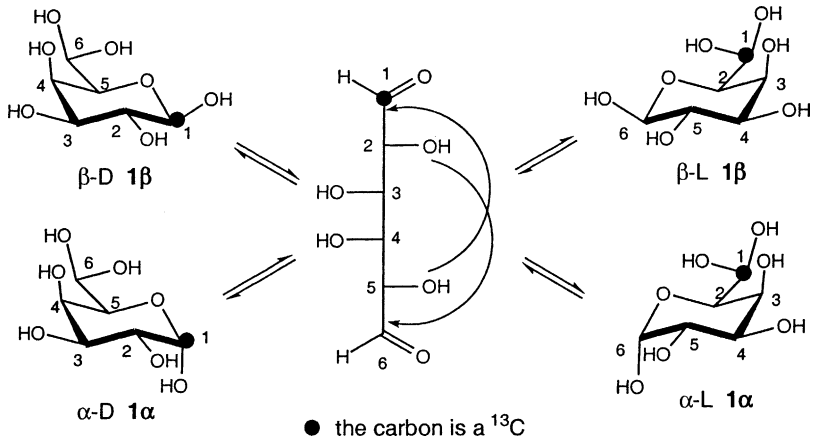
[a] NMR chemical shifts of galactosides from literature data [12–14] are also given for comparison.

Kieboom and co-workers have already demonstrated the reality of this equilibrium by using D-(1 and 2-^{13}C) galactose [8]. These authors, on the basis of the ^{13}C NMR spectrum of the oxidation product obtained with GO, confirmed the existence of two forms and assumed them to be bicyclic hemiacetal forms in water, based on favorable acetal formation (reaction between carbon 6 and OH of carbon 3) and computer calculation of chemical shifts (see Scheme 3).

We have done the same experiment with D-(1-^{13}C)-galactose as substrate. A careful analysis of the ^1H NMR (Fig. 2) and of the proton–carbon correlation

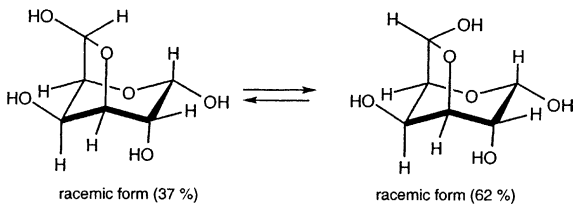


Scheme 1. Hemiacetalic dimers of 6-oxogalactosides.



Scheme 2. Formation of D-[1-^{13}C] and L-[6-^{13}C] hydrate forms of **1**α and **1**β.

NMR spectra (Fig. 3) clearly showed the presence of a racemic mixture of the two enantiomeric D and L forms, but the coupling constants were not consistent with the existence of such bicyclic acetals in aqueous solution. However, the bicyclic nature of 6-oxogalactose (**1**) was recently proven in non-aqueous solvent like ethylene glycol [11]. Thus, proton resonance at 4.57 ppm (see Fig. 2) was directly connected with a proton at 3.49 ppm which presented two large coupling constants (7.8 and 9.8 Hz). As the latter must be necessarily a H-2 proton of β-form, then the former is a H-1 anomeric β-proton.



Scheme 3. Bicyclic hemiacetalic forms proposed for 6-oxogalactose [8].

The proton–carbon correlation allowed the attribution of the ^{13}C spectrum (see Fig. 3). Thus, the D form had a ^{13}C at the 1 position [^{1}H NMR, H-1 β(D) at δ = 4.57 ppm, $^{1}J(^{13}C–H)$ = 161 Hz and H-1 α(D) at δ = 5.26 ppm, $^{1}J(^{13}C–H)$ = 162 Hz; ^{13}C NMR, C-1 β(D) at δ = 99.3 ppm, C-1 α(D) at δ = 95.0 ppm] while the L form had a ^{13}C at the sixth position [^{1}H NMR, H-6 β(L) at δ = 5.12 ppm, $^{1}J(^{13}C–H)$ = 166 Hz and H-6 α(L) at δ = 5.09 ppm, $^{1}J(^{13}C–H)$ = 166 Hz; ^{13}C NMR, C-6 β(L) at δ = 90.9 ppm, C-6 α(L) at δ = 91.2 ppm].

The analysis of the NMR spectra revealed that the main form present for each compound studied was the monomeric hydrate. On standing in water solution, several other minor compounds appeared. It is difficult at the moment to give a correct assignment of the structure of these components, which were assumed to be oligomeric acetals in equilibrium with the hydrate

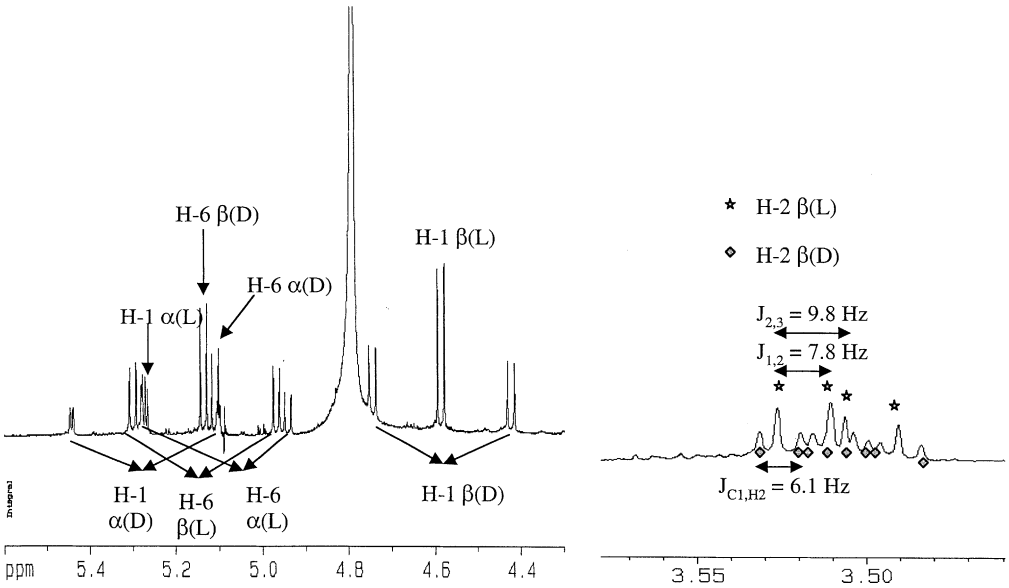


Fig. 2. ^{1}H NMR spectrum of the hydrate form of α- and β-D-[1-^{13}C]*galacto*-hexodialdo-1,5-pyranoside and of α- and β-L-[6-^{13}C]*galacto*-hexodialdo-1,5-pyranoside in D_2O at 500 MHz (TSP, internal reference).

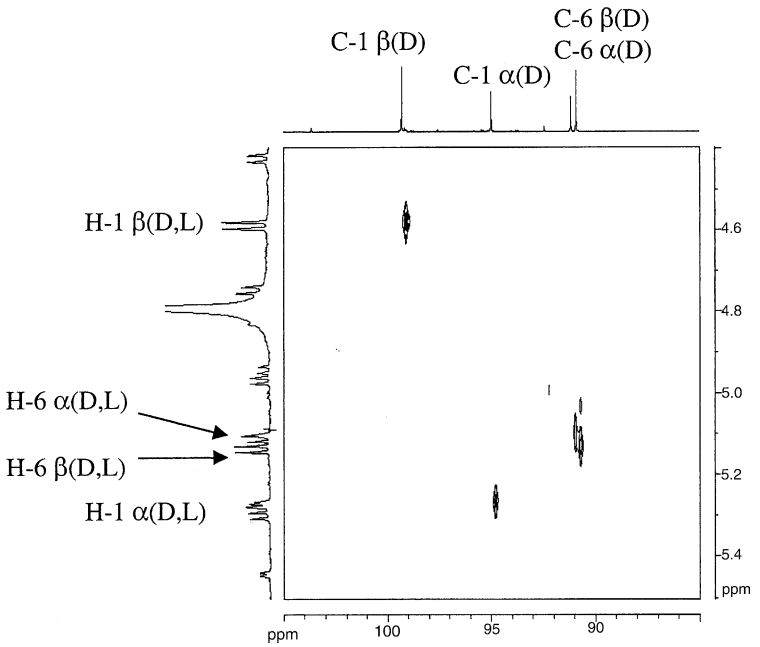


Fig. 3. Proton–carbon NMR correlation (solvent D_2O, TSP, internal reference) of D and L-hydrates obtained by oxidation of D-[1-^{13}C]-galactose (see Scheme 2 for numeration).

Table 5
Effect of substitution of CH_2OH by $CH(OH)_2$ group on ^1H and ^{13}C chemical shifts of galactosides ($\Delta\delta = \delta_{galactoside} - \delta_{6\text{-oxogalactoside}}$ in ppm)

	$\Delta\delta$ H-4	$\Delta\delta$ H-5	$\Delta\delta$ H-6 (approx.)	$\Delta\delta$ C-4	$\Delta\delta$ C-5	$\Delta\delta$ C-6
1α	−0.15	+0.28	−1.39	+0.4	−1.8	−27.4
1β	−0.14	+0.30	−1.42	+0.4	−1.8	−27.3
3	−0.12	+0.31	−1.37	+0.4	−1.8	−27.4
5α/**5**β	−0.13	+0.28	−1.36	+0.6	−1.8	−27.4
6α/**6**β	−0.17	+0.26	−1.38	+0.6	−1.6	−27.0

and the aldehyde. With the aim of providing additional experimental data arguing for the hydrate structure, we have recorded the proton spectra in DMSO d6 hoping for a complexation of the OH, thus resulting in the establishment of a coupling constant of the latter with proton 6. Unfortunately, we were unable to observe this phenomenon since an important shift of the hydrate form to the aldehyde was observed [7,8]. For example the composition of the mixture obtained by oxidation of Me α-D-Gal became in DMSO d6: aldehydic form, 58%; hydrate, 15% and other unidentified components, 27%.

A comparison between the chemical shifts of galactosides [12–14] and those of the hydrates of 6-oxogalactosides (see Table 5) shows that the replacement of CH_2OH group by $CH(OH)_2$ induces:

- chemical shift variation for only protons and carbons at 4, 5 and 6 positions;
- downfield shift for H-6 (1.4 ppm), H-4 (0.14 ppm), C-6 (27.4 ppm) and C-5 (1.8 ppm);
- upfield shift for C-4 (0.4 ppm) and H-5 (0.3 ppm).

The same magnitude of the effects observed whatever the hydrate considered constitutes a useful criterion for the identification of the hydrate form of 6-oxogalactosides.

3. Experimental section

3.1. General procedures

Galactose oxidase from *Dactylium dendroides* and catalase from bovine liver were purchased from Sigma. The chemicals supplied by Sigma were used without any further purification. Deuterium oxide was purchased from Eurisotop (isotopic purity 99.9%). D-(1-^{13}C)-galactose was obtained from Omicron. The course of the reactions was followed by means of TLC (precoated Silica Gel 50 sheets, E. Merck F254) and by ^1H NMR spectroscopy at 500 MHz (Bruker AX500 spectrometer). Three eluents were used: A (30:30:3:3 MeOH–$CHCl_3$–AcOH–H_20); B (30:60:4:4 MeOH–$CHCl_3$–AcOH–H_20); C (40:50:4:4 MeOH–$CHCl_3$–AcOH–H_20). The complete analysis of the ^1H and ^{13}C NMR resonances and the subsequent structure assignments were made using standard 2D sequences (COSY HH and HCOOR correlations) and by comparison with previously published data [13,12]. The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^1H [solvent D_2O, chemical shifts in ppm quoted from the resonance of methyl protons of (trimethylsilyl)-3-propansulfonic acid at 0 ppm) and 126 MHz for ^{13}C (solvent D_2O, chemical shifts in ppm quoted from the resonance of methyl protons of (trimethylsilyl)-3-propansulfonic acid as an internal reference).

3.2. Synthesis of 6-oxogalactosides (*1*–*6*)

At $T = 4\,^\circ C$, galactose or galactoside (1 mmol, 1eq.) was dissolved in 15 ml of distilled water. The galactose oxidase and the catalase from bovine liver (see Table 6) were added to this solution. The reaction medium was saturated with O_2 by blowing bubbles for 3 min in the solution. After 72 h at 4 °C, the substrate was consumed. The course of the reaction was

Table 6
Experimental conditions used for the synthesis of hydrates of 6-oxogalactosides (**1**–**6**)

Compounds	Units[a] of GO (mmol of substrate)	Units[b] of catalase (mmol of substrate)	Eluent for TLC	R_f
1	50	19600	A	0.64
2	50	19600	B	0.47
3	50	19600	B	0.59
4	50	19600	B	0.69
5	100	39200	C	0.55
6	100	39200	A	0.39

[a] One unit will produce a ΔA_{425} of 1.0 min^{-1} at pH 6 (25 °C) in a peroxidase and *o*-tolidine system. Reaction volume: 3.4 ml, light path: 1 cm.

[b] Units defined as number of μmoles of H_2O_2 consumed per minute at pH 7 (25 °C).

followed by means of TLC (Silica gel plates, E. Merck F254, see Table 6 for eluent and R_f). The reaction mixture was then concentrated under reduced pressure.

In the case of 4-O-(β-D-*galacto*-hexodialdo-1,5-pyranosyl)-(1,4)-D-glucose (**6**), the reaction was not complete. This compound was then separated from the remaining lactose by means of silica gel chromatography. Thus, 20 mg of mixture in 300 μl of eluent A (see above) was introduced at the head of a 1 cm o.d. column containing 20 g of silica gel.

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