Synthesis of Deoxy Derivatives of Lactose and their Hydrolysis by β-Galactosidase from *E. Coli*

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Methyl 2-deoxy-α-lactoside, methyl 3-deoxy-β-lactoside, 1,5-anhydro-4-*O*-β-D-galactopyranosyl-D-glucitol and the 2-deoxy and 2,3-dideoxy derivatives of 1,5-anhydro-4-*O*-β-D-galactopyranosyl-D-glucitol have been synthesized by deoxygenation of lactose derivatives at appropriate positions. Cyclohexyl β-D-galactopyranoside has also been synthesized. All derivatives proved to be substrates for the enzyme β-galactosidase from *E. coli*, but the rate of hydrolysis of the substrate analogues was strongly dependent on the nature of the aglycone.

It is well known that carbohydrates play an important role in recognition processes in biological systems, e.g. the blood group determinants.[1] Studies of the binding of oligosaccharides, e.g. those[2] related to the Lewis-b blood group determinants, to lectins and to antibodies have led Lemieux to propose the hydrate polar-gate effect[3] on the strength and specificity of binding of oligosaccharides by proteins.

In a related research program we have investigated carbohydrate–protein interactions by use of chemically modified substrate analogues of methyl β-lactoside[4] in the study of β-galactosidase from *E. coli* (EC 3.2.1.23), for which lactose is the natural substrate. β-Galactosidase has two binding sites,[5–8] namely the galactosyl-site and the glucose-site. In a previous publication[9] we have described the enzymatic hydrolysis of substrate analogues modified in the β-D-galactopyranosyl ring of methyl β-lactoside with respect to the galactosyl-site and in the present work we investigate the specificity of the glucose-site of the enzyme with substrates modified in the reducing unit.

Result and discussion

All unprotected derivatives and key intermediates were analyzed completely by their ^1H and ^{13}C NMR parameters in order to support their structural identity and purity. Furthermore, the NMR data for the unprotected compounds are in excellent agreement with those recently published by Rivera-Sagredo *et al.*[10]

1,5-Anhydro-2-deoxy-4-*O*-β-D-galactopyranosyl-D-*arabino*-hexitol **3** was prepared from the hexa-*O*-acetyl-lactal **1**[11] by high pressure hydrogenation using Pd/C as a catalyst to give 3,6-di-*O*-acetyl-1,5-anhydro-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*arabino*-hexitol **2** in 51 % yield (Scheme 1). Deacetylation of **2** with sodium methoxide in methanol gave crystalline **3** (78 %).

Compound **1** was also used as the starting material for the synthesis of methyl 2-deoxy-α-lactoside **7**. The lactal **1** was reacted with bromine in methanol in the presence of silver acetate following the procedure of Lemieux and Fraser-Reid.[12] The resulting mixture was purified to give methyl 3,6-di-*O*-acetyl-2-bromo-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranoside **4** (65 %) and methyl 3,6-di-*O*-acetyl-2-bromo-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside (**5**, 18 %). Compound **4** was reduced with tributyltin hydride in toluene and then deacetylated to give crystalline methyl 2-deoxy-α-lactoside **7** (40 % overall). Likewise, compound **5** was deacetylated with sodium methoxide to give crystalline methyl 2-bromo-2-deoxy-β-lactoside **8** containing 5 % impurity in the form of a deoxy sugar. Recrystallization did not improve the purity.

Methyl 3-deoxy-β-lactoside **13** was synthesized from 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-hydroxyglucal **9** (Scheme 2).[13] Isomerisation of the double bond by treatment of **9** with boron trifluoride–diethyl ether in anhydrous acetic acid gave 1,2,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-*threo*-hex-2-enopyranose **10** (α:β > 95:5) in quantitative yield. The key step in this synthesis was the hydrogenation of **10** which could give rise to either the *gluco* or the *manno* configuration in the reducing ring. It turned out that treatment of **10** with hydrogen at 3 atm using Pd/C as a catalyst gave only the *gluco* isomer 1,2,6-tri-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranosyl)-α-D-*ribo*-hexopyranose **11** (73 %). Compound **11** was transformed into the corresponding methyl β-glycoside by successive treatment with hydrogen

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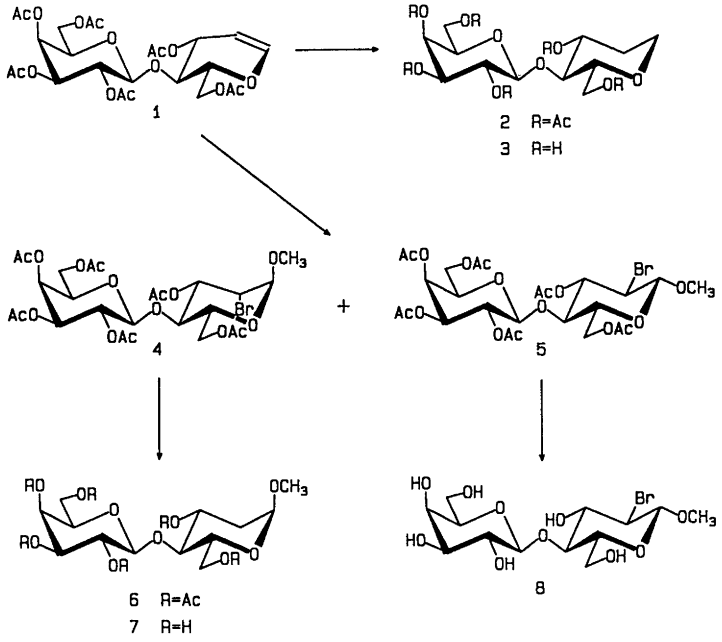
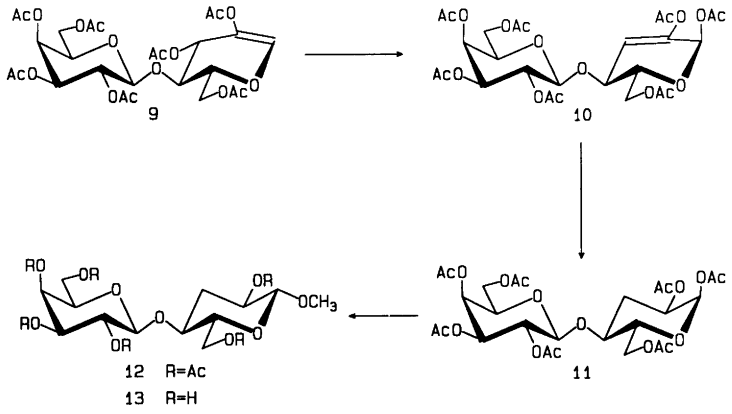


Scheme 1.



Scheme 2.

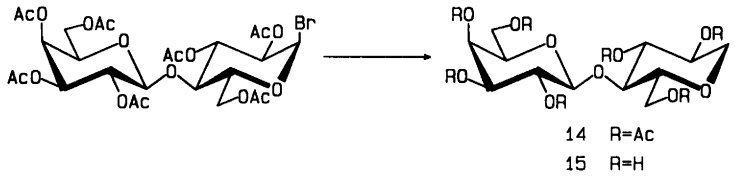
bromide in acetic acid followed by methanol in the presence of silver carbonate to give methyl 2,6-di-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-*ribo*-hexopyranoside **12**, which was subsequently deacetylated with sodium methoxide in methanol to give crystalline methyl 3-deoxy-β-lactoside **13**.

Crystalline 1,5-anhydro-4-*O*-β-D-galactopyranosyl-D-glucitol **15** was prepared in 46% overall yield by high-pressure hydrogenation of hepta-*O*-acetyllactosyl bromide in ethyl acetate and triethylamine using Pd/C as a catalyst followed by deacetylation (Scheme 3).

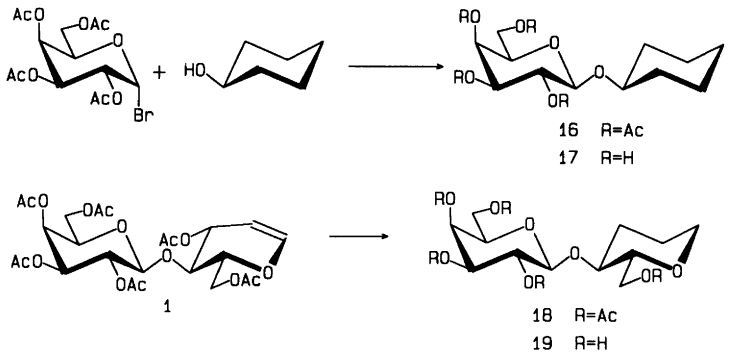
Cyclohexyl β-D-galactopyranoside **17** was synthesized via a conventional glycoside synthesis. Glycosylation of tetra-*O*-acetyl-α-D-galactopyranosyl bromide with four equivalents of cyclohexanol in dichloromethane in the presence of three equivalents of silver carbonate gave cyclohexyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **16**. Deacetylation using sodium methoxide in methanol gave crystalline **17** (65%, Scheme 4).

1,5-Anhydro-2,3-dideoxy-4-*O*-β-D-galactopyranosyl-D-*erythro*-hexitol **19** was prepared from hexa-*O*-acetyllactal **1**[11] by hydrogenation under pressure in diethylamine using Pd/C as a catalyst. The resulting 6-*O*-acetyl-1,5-anhydro-2,3-dideoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*erythro*-hexitol **18** containing product mixture was purified by preparative TLC and the desired compound deacetylated to give crystalline **19** (21%).

The above-mentioned unprotected derivatives of methyl β-lactoside have all been tested as substrates for the enzyme β-galactosidase from *E. coli* by following the progress of hydrolysis by means of ^{1}H NMR spectroscopy.[14] All derivatives proved to be substrates, but the rate



Scheme 3.



Scheme 4.

of hydrolysis was strongly dependent on the aglycone and varied from $t_{1/2}$ = 19 min for cyclohexyl β-D-galactopyranoside **17** to $t_{1/2}$ = 579 min for the dideoxy derivative **19** as seen in Table 1. The experiments were carried out under standard conditions at concentrations that, for lactose, corresponded to 8 × K_M (1.35 mM).[15] Use of NMR spectroscopy to monitor the progress of hydrolysis has the advantage that several substrates can be measured simultaneously,[14] however the determination of K_M values is associated with high uncertainty. We therefore measured the competition between methyl β-lactoside, which was chosen as a reference substrate, and some of the compounds listed in Table 1, using the same experimental conditions as described above. The results are presented in Table 2. From these it can be concluded that all deoxy compounds compete with the hydrolysis of methyl β-lactoside but with different efficiencies as also indicated by the ratio V_{max}/K_M in Table 1. Thus, the $t_{1/2}$ for methyl β-lactoside is increased from 47 min to 96 min when competing with one equivalent of cyclohexyl β-D-galactopyranoside **17**, whereas the $t_{1/2}$ for **17** itself is roughly unchanged. Similar results were observed for compounds **3**, **13**, **7** and **19**, in increasing order with the last-mentioned inhibiting the hydrolysis by more than a factor of 10. These results imply that hydrophobic interactions play an important role in the binding between the aglycone and the glucose-site of the enzyme. However, more detailed kinetic measurements need to be carried out in order to elaborate further the binding of the substrates to the active site of the enzyme.

Experimental

General procedures. ^{1}H NMR spectra were recorded on Bruker AM-500 and Bruker AC-250 instruments at 300 K

Table 1. Kinetic parameters for enzyme hydrolysis.[a]

Compound	S_0/mM	V_0[b]/mM min^{-1}	$t_{\frac{1}{2}}$[b]/min	K_M[b]/mM	V_{max}/mM min^{-1}	(V_{max}/K_M)/min^{-1}	(V_{max}/K_M[c])/min^{-1}
Me β-lac	11.3	0.065	88.2	1.5	0.075	0.05	0.09
3	11.1	0.048	119.5	0.3	0.050	0.2	0.4
13	11.6	0.024	234.0	0.003	0.024	10	18.0
15	11.3	0.145	20.7	41	0.675	0.02	0.03
Me β-lac	11.2	0.121	46.8	1.0	0.132	0.13	
8	11.4	0.110	52.0	1.6	0.119	0.08	
Lactose	11.3	0.241	25.2	1.4	0.270	0.19	
17	11.2	0.292	19.9	0.3	0.301	0.9	
19	11.2	0.009	579.5	0.007	0.010	1.4	
7	11.2	0.026	266.0	0.02	0.027	1.4	

[a] In the first four experiments the enzyme concentration was ca. 0.5 times the concentration in the remaining experiments. the estimated upper limits on the error on the values are: $\Delta S_0 < 1\,\%$, $\Delta V_0 < 2\,\%$, $\Delta t_{\frac{1}{2}} < 2\,\%$, $\Delta K_M > 100\,\%$, $\Delta V_{max} < 2\,\%$. [b] Determined from an integrated Michaelis–Menten equation.[14] [c] Corrected for difference in enzyme concentration by multiplying by (0.121/0.065).

Table 2. Kinetic parameters for competition experiments.[a]

Compound	S_0/mM	V_0/mM min^{-1}		V_{om}[b]/mM min^{-1}	$t_{\frac{1}{2}}$/min	
	Comp.	Single[c]	Comp.[d]		Single[c]	Comp.[d]
3	11.7	0.107[f]	0.073	0.073	56[f]	86
Me β-lac[e]	11.3	0.145	0.029	0.075	41	148
7	11.2	0.024	0.020	0.020	235	281
Me β-lac[e]	11.2	0.111	0.009	0.015	52	354
13	11.1	0.054[f]	0.049	0.049	109[f]	119
Me β-lac[e]	11.3	0.145	<0.001	0.107	41	248
17	11.1	0.292	0.253	0.253	20	25
Me β-lac[e]	11.3	0.121	<0.003	0.093	47	96
19	11.3	0.010	0.010	0.010	580	574
Me β-lac[e]	11.3	0.121	<0.008	0.008	47	638

[a]The errors on the values are: $\Delta S_0 < 1\%$, $\Delta V_0 < 2\%$, $\Delta t_{\frac{1}{2}} < 2\%$, $\Delta V_{om} < 2\%$. [b]Owing to the S-shape of the progress curves in the competition experiments, the maximum observed rate of hydrolysis is presented here. [c]Parameters for single substrate experiments (see the Experimental). [d]Parameters for competition experiments (see the Experimental). [e]The differences in values are due to instability of the enzyme activity of the reference solution, since the measurements are recorded at different times. [f]These values are estimated from the data in Table 1.

[internal $(CH_3)_4Si$] The HDO-peak was used as an internal reference (4.75 ppm) for D_2O solutions. ^{13}C NMR spectra were recorded on Bruker AM-500, AC-250 and WH-90 instruments. $CDCl_3$ was used as an internal reference (76.9 ppm) for $CDCl_3$ solutions and an external reference was used for D_2O solutions (dioxane 67.4 ppm). All NMR data are given in Tables 3–5. Optical rotations were measured on a Perkin Elmer 241 polarimeter. TLC was performed on silica gel 60 F_{254} (Merck). After preparative TLC the products were extracted with ethyl acetate. All reactions in organic solvents were carried out with exclusion of moisture, and solvents for critical reactions were dried over molecular sieves. Concentrations were carried out under disminished pressure at $T < 50$ °C, unless otherwise stated. Melting points are uncorrected. Elemental analysis was performed by Løven A/S microanalytical laboratory.

*1,5-Anhydro-2-deoxy-4-*O*-β-*D*-galactopyranosyl-*D*-arabino-hexitol* **3**. To a solution of hexa-*O*-acetyllactal **1**[11] (1.48 g, 2.6 mmol) in EtOAc (15 ml), was added Pd/C (142 mg), and the mixture hydrogenated at high pressure (77 atm). Filtration through Celite and purification by preparative TLC with EtOAc–hexane 2:1 as the eluant gave, as the main fraction, 3,6-di-*O*-acetyl-1,5-anhydro-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*arabino*-hexitol **2** (760 mg, 51 % yield, NMR data in Tables 3 and 4). Compound **2** was deacetylated with 0.1 M $NaOCH_3$–CH_3OH (1 ml) in CH_3OH (15 ml). Neutralization with Amberlite IR-120 and crystallization from 90 % ethanol gave **3** (328 mg, 78 % yield) as hygroscopic needles. M.p. 190–193 °C, $[\alpha]_D^{25}$ + 27.5° (*c* 1.3, water). NMR data in Tables 4 and 5. Anal. $C_{12}H_{22}O_9 \cdot H_2O$: C, H.

Methyl 2-deoxy-α-lactoside **7** *and methyl 2-bromo-2-deoxy-β-lactoside* **8**. To a solution of hexa-*O*-acetyllactal **1**[11] (1.15 g, 2.1 mmol) in dry CH_3OH (20 ml), was added first silver acetate (590 mg, 3 mmol) and then Br_2 (180 µl, 3 mmol) dropwise over 5 min. After 25 min of stirring, the silver salts were filtered off and the filtrate concentrated and redissolved in $CHCl_3$ (40 ml). The solution was washed successively with $NaHCO_3$ solution (20 ml), 10 % (w/w) $Na_2S_2O_3$ solution (20 ml) and $NaHCO_3$ solution (20 ml), dried over $MgSO_4$ and concentrated to a colorless syrup. Purification by preparative TLC by elution with EtOAc–hexane 1:1 yielded as the fastest moving fraction methyl 3,6-di-*O*-acetyl-2-bromo-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranoside **4** (900 mg, 65 % yield) and as the next fraction methyl 3,6-di-*O*-acetyl-2-bromo-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **5** (250 mg, 18 %) which was characterized from its ^1H NMR data (Table 3). The 2-bromolactoside derivative **5** (85 mg) was dissolved in dry CH_3OH (10 ml) containing 0.1 M $NaOCH_3$–CH_3OH (1 ml) and stirred for 5 h at 30 °C. Neutralization with mixed-bed ion-exchange resin (MB3) and crystallization from ethanol yielded **8** (38 mg). ^1H NMR spectroscopy (500 MHz, Table 5) showed that **8** was contaminated with a small amount (5 %) of a deoxy compound, which could be methyl 2-deoxy-β-lactoside. Recrystallization did not improve the purity.

A solution of compound **4** (366 mg, 0.55 mmol), Bu_3SnH (400 µl, 1.49 mmol) and α,α′-azoisobutyronitrile (30 mg) in dry toluene (8 ml) was boiled (120 °C) under N_2 for 3 h 15 min. Purification by preparative TLC by elution with EtOAc–hexane 1:1 gave methyl 3,6-di-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*O*-acetyl-β-D-galactopyranosyl)-

Table 3. ^{1}H NMR data of protected lactose derivatives.[a]

Compound	Atom Coupling constant													
	H1 $J_{1,2}$	H2 $J_{2,3}$	H3 $J_{3,4}$	H4 $J_{4,5}$	H5 $J_{5,6a}J_{5,6b}$	$H6_a$ $H6_b$ $J_{6a,6b}$		H1′ $J_{1,2}$	H2′ $J_{2,3}$	H3′ $J_{3,4}$	H4′ $J_{4,5}$	H5′ $J_{5,6a}J_{5,6b}$	$H6'_a$ $H6'_b$ $J_{6a,6b}$		Other
1	6.42	4.84	5.41	4.00	4.12–4.23	4.09	4.44	4.66	5.20	5.02	5.36	3.92	4.12	4.23	
	5.6	3.0	5.2	7.5	2.8,7.3	12.0		7.8	10.4	3.5	0.8	–	–	–	
2	3.49	1.69	4.98	3.63	3.47	4.10	4.42	4.60	5.14	4.98	5.36	3.91	4.09	4.17	
	12	12	–	9	2	12		8	10	3	–	6.6	12		
	3.96	1.90–2.20													
			$J_{1,1}$ = 12												
	5.1	–	$J_{2,2}$ = 12												
5	4.46	5.28	3.72	3.89	3.67	4.06–4.20	4.50	4.48	5.09	4.97	5.36	4.06	4.20		OMe = 3.56
	8.7	10.8	9.1		2.1	12.1		7.8	9.6	3.0	0.8	–	–	–	
6	4.77	1.69	5.28	3.67	3.87	4.17	4.49	4.58	5.14	4.98	5.36	3.89	4.08	4.17	OMe = 3.34
	3.8	11.1	8.7	9.9	5.2,2.1	11.9		7.9	10.3	3.4	1.1	7.4,6.2	11.2		
		2.25													
			$J_{2,2}$ = 12.9												
	1.7	5.4													
9	6.60	–	5.68	4.07	4.21–4.29	4.42	4.21	4.65	5.19	5.00	5.37	3.91	4.09	4.17	
	–		4.7	6.6	–	–	–	8.0	10.6	3.5	1.1	7.7,6.1	11.3		
10	6.26	–	6.06	4.40	4.05	4.16	4.26	4.61	5.21	5.02	5.39	3.95	4.11	4.19	
	–		2.1	9.4	5.0,2.1	12.1		7.8	10.4	3.4	1.1	6.6,6.7	11.1		
12	4.33	4.67	1.70	3.66	3.58	4.04	4.31	4.52	5.16	4.99	5.37	3.93	4.09	4.13	OMe = 3.48
	7.8	11.8	11.8	10.0	5.4,1.8	12.0		7.8	10.3	3.2	1.0	6.2,7.2	10.1		
			2.65												
				$J_{3,3}$ = 11.8											
		5.3	4.7												
16	–	–	–	–	–	–	–	4.56	5.19	5.02	5.38	3.91	4.12	4.20	
								7.9	10.4	3.3	1.0	7.0,6.8	11.0		
18	3.33	1.20–1.60	2.25	3.87	3.33	3.91	4.23	4.48	5.11	4.96	5.32	3.87	4.04	4.14	
								7	11	3	3	7			
	3.40	1.20–1.60	–												

[a]Measured at 500 MHz in $CDCl_3$ at 300 K. Coupling constants are observed first-order values.

α-D-*arabino*-hexopyranoside **6** (250 mg, 77 %) as a syrup, which was characterized from its ^{1}H NMR data (Table 3). Compound **6** was deacetylated with 0.1 M $NaOCH_3$–CH_3OH in CH_3OH, neutralized with Amberlite IR-120 and crystallized from ethanol to give **7** (81 mg, 52 %). M.p. 164–166 °C, $[\alpha]_D^{25}$ + 87.3° (*c* 1.0, water). NMR data in Tables 4 and 5. Anal. $C_{13}H_{24}O_{10}$: C, H.

Methyl 3-deoxy-β-lactoside **13**. To a solution of hepta-*O*-acetyl-2-hydroxylactal **9**[13] (1.14 g, 1.84 mmol) in an-

Table 4. ^{13}C NMR data of protected[a] and unprotected[b] lactose derivatives.

Compound	Atom												
	C1	C2	C3	C4	C5	C6	C1′	C2′	C3′	C4′	C5′	C6′	OCH_3
2	100.9	49.6	69.2	77.2	74.0	61.9	100.3	68.9	70.2	66.5	70.7	60.9	55.1
12	102.1	67.8	34.6	74.5	73.9	62.0	101.4	67.9	69.9	66.1	69.9	60.6	55.7
18	77.9	31.4	24.6	76.9	68.7	63.9	102.2	67.9	70.5	66.8	70.9	61.1	–
3	66.1	32.9	71.6	81.5	79.7	61.3	103.8	71.8	73.4	69.3	76.1	61.7	
7	98.8	36.5	67.8	80.5	71.4	60.8	103.7	71.7	73.3	69.3	76.1	61.8	55.2
13	106.0	68.4	38.2	79.1	75.9	61.3	104.5	71.6	73.4	69.3	74.6	61.7	57.7
15	80.4	69.9	70.4	79.9	77.3	62.3	104.2	72.3	73.8	69.8	76.6	61.6	
17[c]	25.9	24.7	33.9	79.5	32.3	24.5	101.7	71.7	73.8	69.5	75.9	61.7	
19	81.4	31.5	25.3	68.1	76.7	61.8	104.4	71.8	73.5	69.3	75.9	61.7	

[a]Measured at 125.77 MHz in $CDCl_3$ at 300 K. [b]Measured at 125.77 MHz in D_2O at 300 K using dioxane as an internal reference (67.4 ppm). [c]The carbon atoms in the cyclohexyl ring are numbered as in the glucose ring in lactose.

hydrous AcOH (10 ml), was added BF_3–Et_2O (0.2 ml) with vigorous stirring. After 1 h of stirring the mixture was diluted with CH_2Cl_2 (50 ml) and washed with H_2O (50 ml + 2×25 ml) and $NaHCO_3$ solution (2×25 ml), dried over $MgSO_4$ and concentrated to a foam (1.22 g). ^1H NMR spectroscopy (Table 3) showed that all starting material had reacted and that only 1,2,6-tri-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-*threo*-hex-2-enopyranose **10** had been formed with more than 95% purity. Compound **10** (1.1 g, 1.78 mmol) was dissolved in EtOAc (15 ml) and hydrogenated (3 atm) using Pd/C as a catalyst. Filtration followed by preparative TLC using EtOAc–hexane as the eluant gave 1,2,6-tri-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-*ribo*-hexopyranose **11** (833 mg, 73% yield) as the main fraction, which was treated with HBr–AcOH in CH_2Cl_2 to give 2,6-di-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-*ribo*-hexopyranosyl bromide (886 ml). The bromide was dissolved in dry CH_3OH (20 ml) and stirred overnight with Ag_2CO_3 (1.22 g, 4.5 mmol) in the dark. The mixture was filtered through charcoal and Celite and then purified by preparative TLC by elution with ether to give methyl 2,6-di-*O*-acetyl-3-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-*ribo*-hexopyranoside **12** (304 mg, 38% from **9**) as a syrup, which was characterized from its ^1H NMR data (Table 3). The product was deacetylated with 0.1 M $NaOCH_3$–CH_3OH (1 ml) in dry CH_3OH (10 ml). The yellow solution was stirred for 16 h, then neutralized with Amberlite IR-120 and concentrated to a syrup (192 mg), which crystallized from ethanol to give **13** (66 mg) as a hygroscopic solid. M.p. 185–186°C, $[\alpha]_D^{25}$ + 3.5° (*c* 0.9, water). NMR data in Tables 4 and 5. Anal. Found: C 45.12; H 6.99. Calc. for $C_{13}H_{24}O_{10}$: C 45.88; H 7.11.

*1,5-Anhydro-4-*O*-β-D-galactopyranosyl-D-glucitol* **15**. A solution of hepta-*O*-acetyl-α-lactosyl bromide (1.16 g, 1.7 mmol) in EtOAc (20 ml) and triethylamine (3 ml) was hydrogenated overnight under pressure using Pd/C as a catalyst to give 2,3,6-tri-*O*-acetyl-1,5-anhydro-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-glucitol **14**. The crude product was deacetylated with $NaOCH_3$–CH_3OH and then neutralized with Amberlite IR-120 to give a syrup, which was crystallized from aqueous ethanol to give 1,5-anhydro-β-D-galactopyranosyl-D-glucitol **15** (248 mg, 46%) m.p. 225° $[\alpha]_D^{25}$ + 48.4° (*c* 1.5, water), lit.[16] m.p. 240°C, $[\alpha]_D$ + 54°. NMR data in Tables 4 and 5.

Cyclohexyl β-D-*galactopyranoside* **17**. To a solution of tetra-*O*-acetyl-α-D-galactopyranosyl bromide (1 g, 2.4 mmol) in CH_2Cl_2 (20 ml) was added cyclohexanol (1 ml, 9.6 mmol) and Ag_2CO_3 ((2 g, 7.2 mmol). The mixture was stirred in a dark sealed flask, then filtered through Celite and charcoal, and concentrated to give a syrup (1.7 g). Purification by column chromatography, using EtOAc–hexane 1:1 as the eluant, gave pure cyclohexyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **16** (1.02 g, 97%) as a syrup. NMR data in Table 3. The acetate **16** (500 mg, 1.1 mmol) was deacetylated overnight with 0.1 M $NaOCH_3$–CH_3OH (2 ml) in CH_3OH (10 ml). Neutralization with Amberlite IR-120 and crystallization from diethyl ether–hexane gave cyclohexyl β-D-galactopyranoside **17** (198 mg, 65%) as a hygroscopic solid. M.p. 130–133°C, $[\alpha]_D^{25}$ – 14.8° (*c* 1.5, water). NMR data in Tables 4 and 5. Anal. $C_{12}H_{22}O_6 \cdot \frac{1}{2}H_2O$: C, H.

*1,5-Anhydro-2,3-dideoxy-4-*O*-β-D-galactopyranosyl-D-erythro-hexitol* **19**. Hexa-*O*-acetyllactal **1** (883 mg, 1.6 mmol) was dissolved in diethylamine (10 ml) and hydrogenated for 3 days under high pressure using Pd/C as a catalyst. Purification by preparative TLC using EtOAc–hexane 2:1 as the eluant gave 6-*O*-acetyl-1,5-anhydro-2,3-dideoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*erythro*-hexitol **18** as a syrup. NMR data in Tables 3 and 4. Deacetylation with 0.1 M $NaOCH_3$–CH_3OH (1.5 ml) in CH_3OH (15 ml) followed by neutralization with Amberlite IR-120 and concentration gave a syrup, which could be crystallized from ethanol to give 19 (95 mg, overall

Table 5. ^{1}H NMR data of unprotected lactose derivatives.[a]

Compound	Atom Coupling constant														
	H1 $J_{1,2}$	H2 $J_{2,3}$	H3 $J_{3,4}$	H4 $J_{4,5}$	H5 $J_{5,6a}J_{5,6b}$	$H6_a$	$H6_b$ $J_{6a,6b}$	H1′ $J_{1,2}$	H2′ $J_{2,3}$	H3′ $J_{3,4}$	H4′ $J_{4,5}$	H5′ $J_{5,6a}J_{5,6b}$	$H6'_a$	$H6'_b$ $J_{6a,6b}$	Other
3	3.53 12,0.1 3.98 4.9,1.2	1.63 12.0 2.06 5.1	3.79 9.2	3.50 9.2	3.41 2.3,5.1	3.95	3.76 11.0	4.45 7.8	3.55 10.0	3.67 3.2	3.93 0	3.74 –	3.71 –	3.81	
7	4.94 3.8 0.9	1.73 12.0 2.20 5.3	3.99 8.8 $J_{2,2}$ = 13.5	3.60 9.1	3.72–3.82 2.1,4.9	3.94	3.88 12.0	4.47 7.9	3.56 10.0	3.68 3.5	3.94 0.8	3.72 –	–	3.82 –	OMe = 3.39
8	4.72 7.9	3.90 10.2	3.68 –	– –	3.80 –	3.84	4.01 12.1	4.49 7.9	3.56 9.8	3.68 3.4	3.94 0.8	3.68 –	– –	3.80 –	OMe = 3.60
13	4.33 8.0	3.47 11.8 4.2	1.66 11.8 2.60 $J_{3,3}$ = 12.0	3.57 –	3.71–3.81 2.0,–	3.79	3.71 –	4.45 8.0	3.49 10.0	3.64 3.4	3.92	3.68 –	3.71 –	3.81 –	OMe = 3.56
15	3.31 11 3.99 5	3.65 –	3.59 9	3.62 9	3.50 5.8	3.79	3.96 12	4.46 8	3.56 10	3.68 3	3.95 1	2.75 –,5	3.77 11	3.82	
17[b]	–	–	–	3.73 –	–	–	–	4.47 8.0	3.41 9.9	3.60 3.6	3.88 0.5	3.62 4.9,7.5	3.72 11.4	3.69	
19	3.45 11.8,2.4 3.94 –	1.60 1.75 –	1.68 2.31 –	3.65 9.5 $J_{1,1}$ = 11.8	3.40 2.7,7.5	3.94	3.75	4.48 7.8	3.51 9.9	3.65 3.5	3.94 0.8	3.68 5.8,7.8	3.75 12.1	3.79	

[a]Measured at 500 MHz in D_2O at 300 K using the DOH signal as a reference (4.75 ppm; acetone 2.225 ppm). Coupling constants are observed first-order values. [b]The protons in the cyclohexyl ring are numbered as those in the glucose ring of lactose. Other observed chemical shifts are 1.08–1.35 ppm five protons, 1.50 ppm two protons, 1.69 ppm two protons and 1.50 ppm one proton.

yield 21 %). M.p. 165–166 °C, $[\alpha]_D^{25}$ + 40.5° (*c* 1.2, water). NMR data in Tables 4 and 5. Anal. $C_{12}H_{22}O_8$: C, H.

Enzymatic procedures. β-Galactosidase (Grade VIII, No G-5636) from *Escherichia coli* (E.C. 3.2.1.23) was obtained from SIGMA. The standard enzyme solution was made by dissolving lyophilized enzyme (1.15 mg) in 0.1 M sodium phosphate buffer (930 µl) then adding 30 mM dithiothreitol in D_2O (35 µl) and 30 mM $Mg(NO_3)_2$ in D_2O (35 µl). The solution was stored at 3–5 °C. The sodium phosphate buffer was prepared by dissolving $NaD_2PO_4 \cdot D_2O$ (345 mg) and $Na_2DPO_4 \cdot 2D_2O$ (445 mg) in D_2O (50.0 ml), to give a pD of 7.2. A standard enzyme experiment was performed by dissolving the appropriate substrate for single-substrate experiments and methyl β-lactoside and the competing substrate in competition experiments in 0.1 M sodium phosphate buffer in D_2O (930 µl) then adding 30 mM dithiothreitol in D_2O (35 µl) and 30 mM $Mg(NO_3)_2$ in D_2O (35 µl). The solution was brought ot 27 °C, a portion (16 µl) of the standard enzyme solution was added and the solution was shaken. The mixture (0.6 ml) was transferred to an NMR tube, which was quickly degassed and placed in the spectrometer probe. After 4–6 min the recording was started. The data were processed by the program

REGGRAFA[14] in order to detemrine the rate constants V_0 and $t_{\frac{1}{2}}$.

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