The Function of the 5-Hydroxymethyl Group of Lactose in Enzymatic Hydrolysis with β-Galactosidase from *E. coli*

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A series of 6-substituted methyl lactoside derivatives together with methyl allolactoside and (6*S*)-methyl [6-^{2}H]lactoside have been synthesized and characterized by NMR spectroscopy. All compounds were tested as substrates for the enzyme β-galactosidase from *E. coli* using progress curve kinetic methology both in single-substrate and competition experiments. The results show that the hydrolysis of methyl lactoside to a large extent takes place through an intramolecular transglycosidation reaction via allolactoside. Furthermore, methyl 6-amino-6-deoxy-D-glucopyranoside proved to be an ihibitor for the enzymatic hydrolysis.

The natural substrate for β-galactosidase from *E. coli* (EC 3.2.1.23) is lactose. However, substantial glycosyl transferase activity is observed which accounts for the production of allolactose [β-D-galactopyranosyl-(1-6)-D-glucopyranose], the natural inducer for the *lac*-operon[1–3] for *E. coli*. Results from Wallenfels *et al.*[4] suggest that the transglycosylation takes place as an intramolecular reaction. However, later studies[5] have shown that allolactose is also produced when a concentrated mixture of D-galactose and D-glucose is treated with β-galactosidase from *E. coli*. Thus both intra- and inter-molecular transglycosylation may therefore arise from the action of the enzyme depending on the reaction conditions. As a continuation of our previous studies[6] we have in the present work investigated the influence of substitutions at the 6-position on the rate of hydrolysis of methyl β-lactoside by β-galactosidase and the mechanism for the transglycosylation reaction.

Results and discussion

The syntheses of the substrates were not optimized as the main objective of the work was the enzymatic results. The structural identity and purity of the unprotected derivatives and key intermediates were assessed by ^{1}H- and ^{13}C-NMR spectroscopy.

Peracetylated 1,6-anhydrolactose **2** was prepared from peracetylated α-lactosyl fluoride which in turn was obtained in quantitative yield from octa-*O*-acetyllactose by treatment with anhydrous HF for a few minutes (Scheme 1). The lactosyl fluoride was heated with KOH in ethanol–water and reacetylated with acetic anhydride in pyridine to give 2,3-di-*O*-acetyl-1,6-anhydro-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*O*-galactopyranosyl)-β-D-glucopyranose **2** which was purified by crystallization in 32 % yield. The above procedure is a viable alternative to literature procedures[7,8] because of the ready availability of the starting glycosyl fluoride.

Methyl 2,3-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **4** is a key intermediate for the synthesis of 6-substituted methyl β-lactoside analogues and it was prepared in 53 % yield following the method of Tejima *et al.*[7] Thus opening of the 1,6-anhydride **2** into 2,3-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-glucopyranosyl chloride with titanium tetrachloride in chloroform followed by glycosylation with silver carbonate in methanol gave **4**. The unprotected 6-hydroxymethyl group was activated towards nucleophilic substitution by mesylation with methanesulfonyl chloride in pyridine.

Methyl 6-azido-6-deoxy-β-lactoside **7** and methyl 6-deoxy-6-iodo-β-lactoside **10** were both prepared by nucleophilic substitution of the mesyl group in methyl 2,3-di-*O*-acetyl-6-*O*-methylsulfonyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **5**. Treatment of **5** with sodium iodide in acetonitrile gave the 6-deoxy-6-iodo analogue **9** in 87 % yield, which upon deacetylation gave methyl 6-deoxy-6-iodo-β-lactoside **10**. Treatment of **5** with sodium azide in dimethylformamide gave the 6-azido-6-deoxy analogue **6** in 69 % yield, which upon deacetylation gave methyl 6-azido-6-deoxy-β-lactoside **7**. Methyl 6-amino-6-deoxy-β-lactoside **8** was obtained by reduction of the unprotected azido compound **7** with H_2S in pyridine in the presence of triethylamine. The triethylamine was observed to be necessary for the completion of the reaction.

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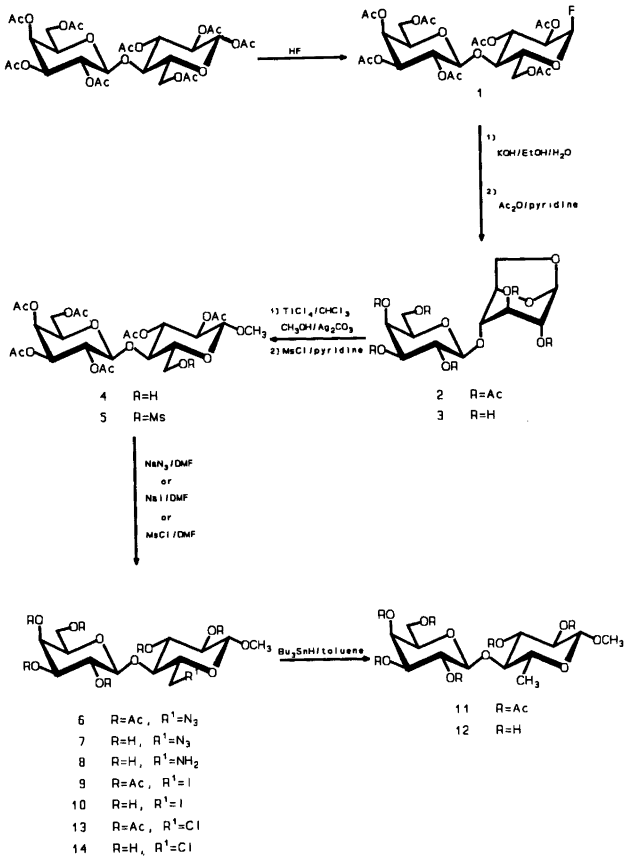
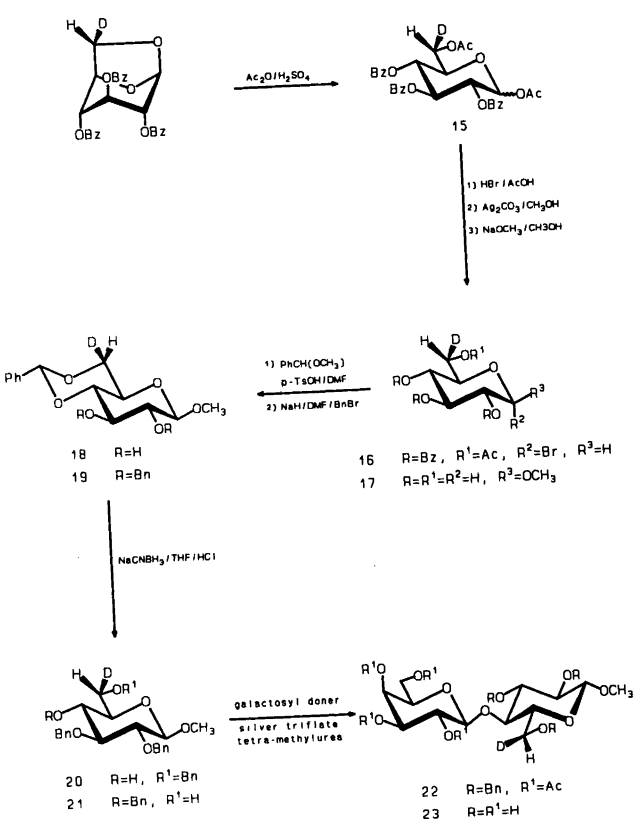


Scheme 1.



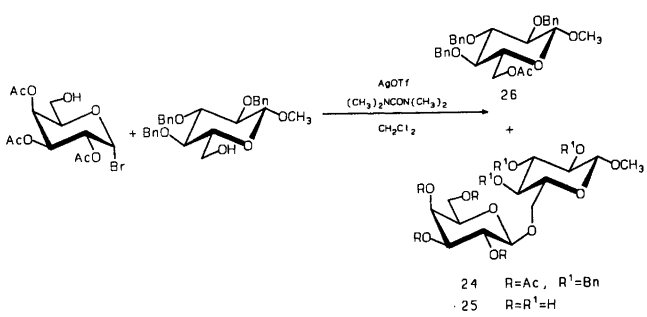
Scheme 2.

Methyl 6-deoxy-β-lactoside **12** was obtained by reduction of the 6-iodo analogue **5** with tributyltin hydride in toluene which gave methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **11** in 70 % yield followed by deacetylation with sodium methoxide.

Treatment of compound **4** with methanesulfonyl chloride in dimethylformamide[9] gave methyl 2,3-di-*O*-acetyl-6-chloro-6-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucoyranoside **13**, which could be deacetylated with sodium methoxide to give methyl 6-chloro-6-deoxy-β-lactoside **14**.

An important compound for the investigation was the selectively 6*S* deuteriated derivative of methyl β-lactoside. Selective photobromination[10] of **2** was not possible owing to the formation of numerous by-products during the reaction. Therefore, this compound had to be prepared by a glycoside synthesis using partially protected methyl (6*S*)-[6-^{2}H]-β-glucoside **17** as aglycone (Scheme 2). Compound **17** was prepared from (6*S*)-1,6-anhydro-2,3,4-benzoyl[6-^{2}H]-D-glucose[11] by simple acid-catalyzed opening of the 1,6-anhydride followed by conversion into the methyl glycoside by reaction with silver carbonate in methanol. Compound **17** was transformed into the aglycone (6*S*)-methyl 2,3,6-tri-*O*-benzyl[6-^{2}H]-β-D-glucopyranoside **20** following the procedure of Garegg *et al.*[12] Compound **20** was then coupled with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide using silver trifluoromethanesulfonate and *N,N,N',N'*-tetramethylurea as a catalyst to give, in 40 % yield, (6*S*)-methyl 2,3,6-tri-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)[6-^{2}H]-β-D-glucopyranoside **22**, which could be deprotected by hydrogenation using Pd/C as a catalyst followed by deacetylation with sodium methoxide to give (6*S*)-methyl [6-^{2}H]-β-lactoside **23**.

Methyl β-allolactoside **25** was synthesized by coupling 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide with methyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside[13] in the presence of silver trifluoromethanesulfonate and *N,N,N',N'*-tetramethylurea to give methyl 2,3,4-tri-*O*-benzyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **24** in 40 % yield (Scheme 3). A by-product which was isolated in 49 % yield was identified as methyl 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside (**16**) and is



Scheme 3.

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

Compound	S_0/mM	V_0[b]/mM min^{-1}	$t_{1/2}$[b]/min	K_M[b]/mM	k_{cat}[b]/10^{-2} min^{-1}	(k_{cat}/K_M)/10^{-2} min^{-1}
Me β-lac	11.3	0.112	52.0	1.0	1.03	1.03
3	11.1	0.064	95.9	3.0	0.66	0.22
8[c]	11.3	0.041	418	–	–	–
10	11.3	0.378	15.1	1.3	3.82	2.94
12	11.3	0.296	20.2	2.3	2.83	1.23
14	11.3	0.374	16.6	2.3	3.73	1.62
23	11.5	0.142	42.7	1.4	1.30	0.92
25	11.2	0.240	26.2	1.7	2.29	1.35

[a]The estimated upper limits on the error on the values are: $S_0 < 1\,\%$, $V_0 < 2\,\%$, $t_{1/2} < 2\,\%$, $K_M \sim 100\,\%$, $V_{max} < 2\,\%$. [b]Determined from an integrated Michaelis–Menten equation.[15] [c]6-Amino-D-glucose is a potent inhibitor, so the reaction stops after ca. 500 min.

most probably formed by transacetylation of the aglycone.[14]

The synthesized unprotected sugars were all tested as substrates for β-galactosidase from *E. coli* (EC 3.2.1.23) using methyl β-lactoside as the reference substrate. The enzymatic reactions were monitored by ^{1}H NMR spectroscopy as described in a previous publication.[15] All analogues proved to be substrates, although the hydrolysis of methyl 6-amino-6-deoxy-β-lactoside **8** seems to stop before completion (Table 1) because the released methyl 6-amino-6-deoxy-β-D-glucopyranoside is an inhibitor of the enzyme (Table 2). Substitution of the 6-hydroxy group with a deoxy function or a halide gave analogues which were all hydrolysed at more than double the rate of methyl β-lactoside, but introducing a 1,6-anhydro bridge gave a more slowly hydrolysed substrate. The reason for the last result is probably that the affinity for the substrate was decreased owing to the inverted conformation of the glucopyranose ring.

The above results together with competition experiments between the substrates (Table 2) suggest that if the substrate contains a 6-hydroxy group, this group will, to a large extent, be directly involved in the enzymatic hydrolysis, presumably due to the formation of a β-(1-6) linked transglycosylation product (allolactose). It is furthermore important to notice that a deuterium effect of the magnitude observed for (6*S*)-methyl [6-^{2}H]-β-lactoside **23**, which was hydrolysed faster than the non-deuteriated reference, only seems possible if direct involvement of the 6-hydroxy group occurs during the hydrolysis.

The transglycosylated product can furthermore be detected by NMR spectroscopy during a normal enzyme reaction, but it never exceeds more than about 5 % of the total concentration owing to the enhanced hydrolysis rate of methyl β-allolactoside **25**. The configuration of the transglycosylation product from the hydrolysis of (6*S*)-methyl [6-^{2}H]-β-lactoside **23** was analysed by NMR spectroscopy

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

Compound	S_0/mM	V_0/mM min^{-1}		V_{om}[b]/mM min^{-1}	$t_{1/2}$/min	
		Single[c]	Comp.[d]		Single[c]	Comp.[d]
3	11.3	0.074	0.009	0.046	80	196
Me β-lac	11.3	0.121	0.101	0.101	47	59
8	11.4	0.042	0	0	(418)	∞
Me β-lac	11.2	0.112	0.103	0.103	51	71
12	11.4	0.344	0.124	0.124	18	49
Me β-lac	11.2	0.121	<0.055	0.097	47	75
14	11.2	0.401	0.188	0.188	15	35
Me β-lac	11.3	0.121	0.083	0.083	47	70
25	11.3	0.256	0.163	0.163	23	38
Me β-lac	11.3	0.121	<0.034	0.087	47	93
12	11.3	0.344	0.036	0.141	18	65
25	11.3	0.256	0.205	0.205	23	31
Me 6-NH_2-Glc[e]	13.3	0	0	0	∞	∞
Me β-lac	11.3	0.112	0.062	0.062	51	723

[a]The error on the values are: $S_0 < 1\,\%$, $V_0 < 2\,\%$, $t_{1/2} < 2\,\%$, $V_{om} < 2\,\%$. [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]Methyl 6-amino-6-deoxy-β-D-glucopyranoside.

Table 3 (a). Proton NMR data in $CDCl_3$ (ppm values).

	H1	H2	H3	H4	H5	$H6_a$	$H6_b$	H1′	H2′	H3′	H4′	H5′	$H6'_a$	$H6'_b$	OCH_3
2	5.46	5.16	4.55	3.57	4.60	3.81	3.99	4.81	5.29	5.04	5.39	4.00	4.06	4.16	
4	4.43	4.86	5.20	3.96	3.41	3.76	3.92	4.65	5.11	5.01	5.35	3.94	4.08–4.18		3.49
5	4.43	4.87	5.22	3.88	3.65	4.36	4.58	4.63	5.13	4.99	5.36	3.92	4.08	4.14	3.50
6	4.45	4.91	5.20	4.11	3.82	3.23–3.33		4.52	5.14	4.99	5.35	3.96	3.55–3.72		3.50
9	4.45	4.90	5.21	3.68	3.22–3.34		3.59	4.63	5.14	4.99	5.35	3.90	4.09	4.13	3.50
11	4.35	4.88	5.14	4.00	3.46	——1.37——		4.75	5.14	5.00	5.35	3.88	4.09	4.13	3.48
13	4.46	4.88	5.21	3.93	3.67	3.75	3.88	4.65	5.11	5.01	5.35	3.92	4.09	4.12	3.51
19	4.41	3.45	3.75	3.68	3.41	4.34		PHC*H*=5.57							3.58
20	4.32	3.40	3.45	3.58	3.44	3.68									3.56
21	4.35	3.40	3.67	3.56	3.36	3.70									3.56
22	4.28	3.40	3.95	3.57	3.36	3.71	–	4.63	5.11	4.82	5.25	3.53	4.01	3.85	3.57
24	4.29	3.40	3.64	3.36	3.51	4.12	4.12	4.61	5.36	4.98	5.38	3.87	4.12	3.66	3.58
26	4.31	3.44	3.68	3.55	3.51	4.24	4.34								3.56

Table 3 (b). Proton NMR J_3 coupling data in $CDCl_3$ (Hz).

	J_{12}	J_{23}	J_{34}	J_{45}	J_{56}	J_{66}	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{5'6'}$	$J_{6'6'}$
2	–	–	1.1	–	5.8,1	7.4	8.0	10.7	3.2	0.8	6.5,6.2	10.8
4	8.0	9.7	9.5	9.5	3.1,–	12.0	7.7	10.3	3.2	0.8	–	–
5	7.8	9.8	9.5	9.1	3.6,2.0	11.9	7.9	10.4	3.6	1.0	7.0,6.5	11.4
6	7.8	9.5	8.5	7.5	–	–	8.0	10.2	3.6	1.0	7.0,–	–
9	7.8	9.5	9.5	9.0	–	–	8.0	10.2	3.6	1.0	–	11.0
13	7.9	9.1	9.1	9.4	4.4,2.2	12.0	7.9	10.4	3.5	0.7	7.2,5.3	11.0
19	7.9	8.5	9.0	9.2	4.9	–						
20	7.8	8.5	9.5	9.5	5.4	–						
21	7.9	9.0	9.0	9.5	4.7	–						
22	7.8	9.1	9.2	9.1	3.9	–	7.7	10.5	3.2	1.2	8.0,5.9	11.2
24	7.9	9.0	9.0	9.4	–	–	8.0	10.4	3.4	0.9	7.0,7.0	11.0
26	7.8	9.2	9.1	9.0	4.0,1.8	12.1						

and found to be 6*S*, which proves that the 6-hydroxy group of glucopyranose attacks the anomeric carbon in the galactosyl–enzyme complex during the transglycosylation.

To substantiate the importance of transglycosylation to the overall rate of hydrolysis it was demonstrated that a large fraction takes place as an intramolecular reaction. This was done by following the enzymatic hydrolysis of (6*S*)-methyl [6-^{2}H]-β-lactoside **23** in the presence of one equivalent of methyl β-D-glucopyranoside. At the beginning of the reaction the ratio of methyl β-glucopyranoside to (6*S*)-methyl [6-^{2}H]glucopyranoside in the solution was close to infinity, which means that the transglycosylation product had to be non-deuteriated if the reaction was intermolecular. However, only the deuteriated transglycosylation product could be detected by NMR spectroscopy, so the reaction has to be predominantly intramolecular. Because of the small amount of transglycosylation product present in the mixture the detection limit for non-deuteriated transglycosylation product was about 10–20%, but this still means that more than 80% of the transglycosylation takes place as an intramolecular reaction.

Table 4. Carbon NMR data in $CDCl_3$ (ppm values).

	C1	C2	C3	C4	C5	C6	C1′	C2′	C3′	C4′	C5′	C6′	OCH_3
2	98.9	69.0	68.4	76.4	73.4	64.7	100.7	68.8	70.8	66.9	71.0	61.2	
4	100.8	71.5	72.8	74.7	74.7	60.1	101.5	69.1	70.4	66.6	70.8	60.7	57.7
5	100.5	71.3	72.5	75.4	72.3	67.0	101.3	69.0	70.5	66.4	70.7	60.5	57.1
6	100.7	71.4	72.6	74.2	73.3	50.1	101.0	69.0	70.5	66.5	70.6	60.6	56.5
9	100.9	71.5	72.2	79.4	73.4	4.4	100.7	69.1	70.6	66.5	70.7	60.6	
13	100.9	71.4	72.7	76.1	73.3	43.0	100.5	69.0	70.4	66.4	70.6	60.5	56.6
20	104.7	81.7	83.9	71.4	73.9	69.8							57.0
22	104.6	81.6	82.4	76.4	73.4	67.3	100.0	69.6	70.3	66.7	70.9	60.5	56.9
24	104.5	82.1	84.3	77.4	74.4	68.1	101.1	68.6	70.5	66.8	70.9	61.1	57.0
26	104.6	82.0	84.4	77.2	72.6	62.9							57.1

Table 5(a). Proton NMR data in D_2O (ppm values).

	H1	H2	H3	H4	H5	$H6_a$	$H6_b$	H1′	H2′	H3′	H4′	H5′	H6′$_a$	H6′$_b$	OCH_3
3	5.47	3.56	3.90	3.85	4.80	4.11	3.80	4.55	3.59	3.68	3.94	3.70	3.81	3.76	
7	4.39	3.28	——— 3.58 → 3.75 ———					4.38	3.49	3.58–3.75	3.87	—— 3.58 → 3.75 ——			3.52
10	4.45	3.29	——— 3.28 → 3.72 ———					4.52	3.28–3.72		3.89	—— 3.28 → 3.72 ——			3.54
12	4.38	3.31	3.58	3.40	3.64	1.38		4.50	3.55	3.66	3.93	3.70–3.79	3.80	3.70–3.79	3.55
14	4.48	3.34	3.81	3.68	3.87	3.73–3.83		4.54	3.56	3.69	3.95	3.73–3.83	4.00	4.04	3.59
17	4.43	3.26	3.49	3.38	3.46	3.70									3.57
23	4.41	3.32	3.64	3.60	3.60	3.80	–	4.45	3.55	3.67	3.93	3.73	3.77	3.80	3.58
25	4.00	3.29	3.50	3.50	2.64	4.24	3.88	4.46	3.57	3.67	3.94	3.71	3.77	3.81	3.59

Table 5(b). Proton NMR J_3 coupling data in D_2O (Hz).

	J_{12}	J_{23}	J_{34}	J_{45}	J_{56}	J_{66}	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{5'6'}$	$J_{6'6'}$
3	–	–	–	–	–	8.0	7.9	10.0	3.2	0.8	8.0,4.1	12.0
12	8.0	9.4	9.3	9.3	6.1	–	8.0	10.0	3.7	0.8	–	–
14	8.0	9.2	9.4	9.1	–	–	7.9	9.7	3.0	0.8	–	11.0
17	8.0	9.6	9.3	9.5	6.1	–						
23	8.0	9.5	9.8	–	5.2	–	7.9	10.0	3.4	0.2	4.5,4.5	8.0
25	7.9	–	–	–	2.6,5.6	11.6	7.9	9.8	3.4	0.8	4.5,8.0	11.6

Experimental

General procedures. ^1H NMR, spectra were recorded on Bruker AM-500 and Bruker AC-250 instruments at 300 K [Internal $(CH_3)_4Si$]. Acetone was used as an internal reference (2.25 ppm) for D_2O solutions. ^{13}C NMR spectra were recorded on Bruker AM-500, AC-250 and WH-90. $CDCl_3$ was used as internal reference (76.9 ppm) for $CDCl_3$ solutions and an external instrument reference was used for D_2O solutions (dioxane 67.4 ppm). All NMR data are given in Tables 3–6. 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 the exclusion of moisture, and solvents for critical reactions were dried over molecular sieves. Concentrations were carried out at dimished pressure at < 50 °C, unless otherwise stated. Melting points measured on a home-built apparatus using a heated oil bath are uncorrected and are for known compounds not carried out to constant melting point because the compounds are further characterized by optical rotations and by extensive NMR analysis. Elemental analysis was performed by Løven A/S microanalytical laboratory.

2,3-Di-O-acetyl-1,6-anhydro-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactosyl)-β-D-glucopyranose **2**. Octa-*O*-acetyllactose[16] (15 g) was dissolved in anhydrous HF (18 ml, 0 °C). After 10 min the reaction was diluted with CH_2Cl_2 and quenched by being poured into ice water. The organic phase was washed successively with water and saturated $NaHCO_3$ solution, dried with $MgSO_4$ and concentrated. The crude 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-glucopyranosyl fluoride **1** (pure according to a ^1H NMR spectrum) was dissolved in a hot mixture of ethanol (65 ml) and a solution of KOH (20 g) in water (80 ml) and heated on a steambath for 0.5 h in a polyethylene vial. The resulting brown solution was neutralized with conc. HCl and concentrated. After removal of traces of water by co-evaporation with acetic acid the product was acetylated with acetic anhydride (70 ml) in pyridine (70 ml). The product mixture was poured into ice–water (200 ml), stirred for 1 h and then extracted with CH_2Cl_2 (150 ml). The organic phase was washed successively with 4 M

Table 6. Carbon NMR data in D_2O (ppm values).

	C1	C2	C3	C4	C5	C6	C1′	C2′	C3′	C4′	C5′	C6′	OCH_3
3	102.2	70.7	72.2	78.4	74.8	65.9	102.8	71.5	73.3	69.4	76.1	61.9	
7	103.9	73.6	74.5	80.1	75.0	51.2	103.9	71.7	73.3	69.3	76.2	61.9	58.0
10	103.8	73.7	73.7	82.7	74.7	6.8	103.8	71.8	73.3	69.3	76.3	61.8	58.3
12	103.8	73.8	75.0	84.5	71.7	17.4	103.9	71.8	73.4	69.4	76.1	61.8	58.0
14	103.8	73.6	74.9	79.5	74.0	42.6	104.0	71.8	73.3	69.4	76.4	61.8	58.3
23	103.8	73.6	75.2	79.3	75.6	60.6	103.9	71.8	73.4	69.4	76.2	61.9	58.0
25	104.2	73.8	76.4	70.1	75.7	69.2	104.2	71.5	73.4	69.4	76.0	61.8	58.2

HCl, saturated $NaHCO_3$ solution, and water, dried with $MgSO_4$ and filtered through charcoal. Concentration and crystallization from ethyl acetate gave **2** (4.15 g, 32 %), m.p. 202–204 °C, $[\alpha]_D^{25}$ −38.0° (*c* 1.0 $CHCl_3$); lit.[7] m.p. 208 °C $[\alpha]_D^{29}$ −39.5°. For NMR data see Tables 3 and 4.

1,6-Anhydrolactose **3**. Compound **2** (406 mg, 0.70 mmol) was deacetylated in methanol (10 ml) and 0.1 M $NaOCH_3$–CH_3OH (1 ml), neutralized with ion-exchange Amberlite IR-120, and crystallized from ethanol to give **3** (115 mg, 51 %), m.p. 126–130 °C, $[\alpha]_D^{25}$ −48.2° (*c* 1.3, water); Lit.[17] m.p. 140–144 °C, $[\alpha]_D$ −53.5°. For NMR data see Tables 5 and 6.

Methyl 2,3-Di-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **4**. Hexa-*O*-acetyl-1,6-anhydrolactose **2** (500 mg, 0.87 mmol) was dissolved in dry $CHCl_3$ (6 ml) and ethanol (0.1 ml) with the exclusion of moisture and cooled in an ice bath. $TiCl_4$ (462 µl, 4.2 mmol) was added and when gas evolution ceased, the mixture was heated to reflux for 4 h and then poured into water (30 ml) together with $CHCl_3$ (15 ml). The organic phase was washed with water (2×20 ml), dried with $CaCl_2$ and concentrated to give 2,3-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-glucopyranosyl chloride as a yellow syrup.

To a solution of the syrup in CH_2Cl_2 (5 ml) was added CH_3OH (15 ml) and Ag_2CO_3 (1.11 g, 4.0 mmol) and the mixture was stirred overnight in a dark sealed flask. The reaction mixture was filtered through Celite and charcoal and concentrated and purified by preparative TLC using ethyl acetate–hexane 3:1 as the eluent. The main fraction was collected as a syrup which crystallized from diethyl ether–hexane to give **4** (278 mg, 53 %), m.p. 146–149 °C, $[\alpha]_D^{25}$ −23.4° (*c* 1.0, $CHCl_3$). For NMR data see Tables 3 and 4.

Methyl 2,3-di-O-acetyl-6-O-methylsulfonyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **5**. To a solution of **4** (350 mg, 0.58 mmol) in pyridine (10 ml) was added methanesulfonyl chloride (0.5 ml) at −10 °C. The mixture was stirred overnight with exclusion of moisture, then water (20 ml) was added and the stirring was continued for a further 1 h. The mixture was extracted with CH_2Cl_2 (60 ml) and the organic phase was washed with 4 M HCl (2×25 ml) and saturated $NaHCO_3$ solution (20 ml), dried with $MgSO_4$ and concentrated. Crystallization from ethanol gave **5** (327 mg, 83 %), m.p. 107–111 °C, $[\alpha]_D^{25}$ −11.1° (*c* 1.2, $CHCl_3$); lit.[18] m.p. 113–114 °C, $[\alpha]_D^{21}$ −14.9°. For NMR data see Tables 3 and 4.

Methyl 2,3-di-O-acetyl-6-azido-6-deoxy-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **6**. Compound **5** (400 mg, 0.6 mmol) was dissolved in DMF (10 ml) and stirred with NaN_3 (400 mg) for 16 h (100 °C). The mixture was filtered and the filtrate poured into H_2O and stirred for 1 h. The white precipitate was filtered off, redissolved in CH_2Cl_2, washed with H_2O, dried with $MgSO_4$, and concentrated to a syrup that crystallized from ethanol to give **6** (260 mg, 69 %) as needles, m.p. 190–191 °C, $[\alpha]_D^{25}$ +7.4° (*c* 1.0, $CHCl_3$); lit.[18] m.p. 200–201 °C, $[\alpha]_D$ +6°. For NMR data see Tables 3 and 4.

Methyl 6-azido-6-deoxy-β-lactoside **7**. Compound **6** (140 mg, 0.2 mmol) was dissolved in CH_3OH (10 ml) and 0.1 M $NaOCH_3$–CH_3OH (1.5 ml) and stirred overnight. The mixture was neutralized with Amberlite IR-120, concentrated and crystallized from ethanol to give **7** (70 mg, 85 %), m.p. 170–171 °C, $[\alpha]_D^{25}$ +3.8° (*c* 0.5, H_2O). For NMR data see Tables 5 and 6. Anal. $C_{13}H_{23}N_3O_{10}$: C, H.

Methyl 6-amino-6-deoxy-β-lactoside **8**. Methyl 6-azido-6-deoxy-β-lactoside **7** (23 mg, mmol) was dissolved in pyridine (10 ml) and triethylamine (0.5 ml) was added. Hydrogen sulfide generated from FeS and H_2SO_4 was bubbled through the solution for 5 h. The dark brown reaction mixture was concentrated and then suspended in water and filtered. The filtrate was concentrated and purified on a Sephadex G-15 column using methanol–water 1:1 as the eluent to give methyl 6-amino-6-deoxy-β-lactoside **8** which was stored as the hydrochloride. Owing to the small amount, the product was characterized only by NMR spectroscopy (Tables 5 and 6).

Methyl 2,3-di-O-acetyl-6-deoxy-6-iodo-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **9**. Compound **4** (327 mg, 0.54 mmol) was dissolved in dry CH_3CN (8 ml) and reacted with NaI (250 mg, 1.67 mmol) on being heated to reflux for 8 h. After cooling the mixture was filtered and concentrated. The resulting dry compound was triturated with water (10 ml), and the yellow precipitate filtered off and recrystallized from ethanol–water to give **9** (298 mg, 87 %), m.p. 159–160 °C, $[\alpha]_D^{25}$ −10° (*c* 1.0, $CHCl_3$); lit.[9] m.p. 166–167 °C, $[\alpha]_D^{22}$ −10°. For NMR data see Tables 3 and 4.

Methyl 6-deoxy-6-iodo-β-lactoside **10**. Compound **9** (170 mg, 0.2 mmol) was deacetylated by reaction overnight with 0.1 M $NaOCH_3$–CH_3OH (1.5 ml) in CH_3OH (10 ml). The mixture was neutralized with ion-exchange Amberlite IR-120, concentrated and crystallized from ethanol to give **10** (75 mg, 68 %), m.p. 126–128 °C, $[\alpha]_D^{25}$ −10.6° (*c* 0.5, water). For NMR data see Tables 5 and 6.

Methyl 6-deoxy-β-lactoside **12**. Compound **9** (200 mg, 0.27 mmol) was dissolved in dry toluene and *n*-Bu_3SnH (150 µl, 0.56 mmol) was added under N_2. After 4.25 h of stirring the mixture was concentrated and washed with hexane. The resulting white powder was purified by preparative TLC by elution with ethyl acetate–hexane 2:1 to give methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **11** (116 mg, 70 %) as a syrup.

Compound **11** was deacetylated in dry methanol by addition of 0.1 M $NaOCH_3$–CH_3OH and stirring for 16 h. The mixture was neutralized with ion-exchange Amberlite IR-120 and concentrated. Crystallization from 95 % ethanol gave methyl 6-deoxy-β-lactoside **12** (48 mg, 64 %), m.p. 229–232 °C, $[\alpha]_D^{25}$ −4.2° (*c* 0.9, water). For NMR data see Tables 5 and 6. Anal. $C_{13}H_{24}O_{10}$: C, H.

Methyl 6-chloro-6-deoxy-β-lactoside **14**. to a solution of **4** (275 mg, 0.45 mmol) in dry DMF (6 ml) was added methanesulfonyl chloride (0.4 ml, 5.28 mmol). The mixture was heated (70 °C) for 16 h with exclusion of moisture, then concentrated (1 mmHg) and purified by preparative TLC in diethyl ether to give methyl 2,3-di-*O*-acetyl-6-chloro-6-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **13** (220 mg, 77 %) as a syrup.

Compound **13** (220 mg, 0.35 mmol) was dissolved in anhydrous CH_3OH (10 ml) containing 0.1 M $NaOCH_3$–CH_3OH (2 ml) and stirred overnight. The resulting crystalline methyl 6-chloro-6-deoxy-β-lactoside (65 mg) was filtered off and washed with cold CH_3OH. The combined filtrate was neutralized with Amberlite IR-120, concentrated and crystallized from ethanol to give more **14** (49 mg, total yield 114 mg, 87 %), m.p. 190–191 °C, $[\alpha]_D^{25}$ −0.7° (*c* 0.6, water). For NMR data see Tables 5 and 6. Anal. $C_{13}H_{23}ClO_{10}$: C, H.

(6S)-*Methyl [6-²H]-β-D-glucopyranoside* **17**. To a suspension of (6*S*)-1,6-anhydro-2,3,4-tri-*O*-benzoyl[6-²H]-β-D-glucopyranose[11] (1.2 g, 2.5 mmol) in Ac_2O (24 ml) was added conc. H_2SO_4 (5 drops). After 4 min at 40 °C the now homogeneous mixture was poured into water (60 ml) and stirred for 1.5 h. The mixture was extracted with CH_2Cl_2 (3×20 ml) and the organic phase was washed successively with H_2O and saturated $NaHCO_3$ solution, dried with $MgSO_4$ and concentrated to give (6*S*)-1,6-di-*O*-acetyl-2,3,4-tri-*O*-benzoyl[6-²H]-D-glucopyranose **15** in quantitative yield.

Crude **15** was dissolved in CH_2Cl_2 (20 ml) and HBr in acetic acid (20 ml) added. After being stirred for 1 h, the mixture was diluted with cold CH_2Cl_2 (20 ml) and washed successively with ice–water and saturated $NaHCO_3$ solution, dried with $MgSO_4$ and concentrated to give (6*S*)-6-*O*-acetyl-2,3,4-tri-*O*-benzoyl[6-²H]-α-D-glucopyranosyl bromide **16** (1.48 g, 97 %) as a syrup.

To a solution of **16** in CH_2Cl_2 (20 ml) was added CH_3OH (20 ml) and Ag_2CO_3 (2.1 g, 7.5 mmol). The mixture was stirred overnight in a dark, sealed flask, then filtered through Celite and charcoal and concentrated to a foam. The crude (6*S*)-methyl 6-*O*-acetyl-2,3,4-tri-*O*-benzoyl[6-²H]-β-D-glucopyranoside was suspended in CH_3OH (20 ml) containing 0.1 M $NaOCH_3$–CH_3OH (2 ml) and stirred over a weekend. The homogeneous mixture was neutralized with Amberlite IR-120 and concentrated to a residue which was fractionated between water and hexane. The water phase was washed with hexane, then concentrated to dryness and crystallized from ethanol to give (6*S*)-methyl [6-²H]-β-D-glucopyranoside **17** (316 mg, 62 % overall), m.p. 98–102 °C; lit.[19] methyl β-D-glucopyranoside m.p. 104–105 °C. For NMR data see Table 5.

(6*S*)-*Methyl 2,3-di-O-benzyl-4,6-O-benzylidene[6-²H]-β-D-glucopyranoside* **19**. Deuteriated methyl β-D-glucopyranoside **17** (276 mg, 1.4 mmol) was dissolved in dry DMF (10 ml) with *p*-TsOH (40 mg) and α,α-dimethoxytoluene (1 ml, 6.6 mmol) and heated to 50 °C under reduced pressure (30 mmHg) on a rotary evaporator for 130 min. The mixture was neutralized with triethylamine and concentrated to dryness by coevaporation with toluene. The product was crystallized from ethanol to give methyl (6*S*)-4,6-*O*-benzylidene[6-²H]-β-D-glucopyranoside **18** (294 mg, 73 %).

To a solution of **18** (276 mg, 0.98 mmol) in dry DMF (7 ml) was added a 55–60 % NaH suspension (500 mg, 11 mmol) in dry DMF (7 ml) and the mixture was stirred for 1 h with exclusion of moisture. A solution of benzyl bromide (1.5 ml, 13 mmol) in DMF (2 ml) was added dropwise and the stirring was continued for 16 h. The reaction was quenched by being cooled to 0 °C and treated with H_2O (30 ml) followed by neutralization with conc. HCl. The product was extracted with CH_2Cl_2 (3×20 ml) and the organic phase was washed with H_2O (3×15 ml) and concentrated (1 mmHg). The residue was extracted with boiling diethyl ether (2×10 ml) and the diethyl ether phase was concentrated and crystallized from hexane to give **19** (392 mg, 87 %), m.p. 113–116 °C, $[\alpha]_D^{25}$ −32.7° (*c* 1.2, $CHCl_3$); lit.[20] m.p. 119–120 °C, $[\alpha]_D^{20}$ −35.8°. For NMR data see Table 3.

(6*S*)-*Methyl 2,3,6-tri-O-benzyl[6-²H]-β-D-glucopyranoside* **20**. To a solution of **19** (392 mg, 0.85 mmol) and $NaCNBH_3$ (1 g, 16 mmol) in THF (15 ml, dried with molecular sieves) was added dropwise HCl in dry ether until gas evolution ceased. The mixture was stirred for 5 min then diluted with CH_2Cl_2 (50 ml), washed successively with H_2O and saturated $NaHCO_3$ solution, dried with $MgSO_4$ and concentrated. Purification by preparative TLC using ethyl acetate–hexane 1:2 as the eluent gave **20** (164 mg, 42 %), m.p. 68–70 °C, $[\alpha]_D^{25}$ −14.8° (*c* 1.7, $CHCl_3$) (lit.[12] m.p. 64–65 °C, $[\alpha]_D^{22}$ −17°) and (6*S*)-methyl 2,3,4-tri-*O*-benzyl[6-²H]-β-D-glucopyranoside **21** (20 mg, 5 %), m.p. 77–80 °C, $[\alpha]_D^{25}$ +7.4° (*c* 1.0, $CHCl_3$) (lit.[13] m.p. 83–85 °C, $[\alpha]_D$ 8.5°). For NMR data see Tables 3 and 4.

(6S)-*Methyl [6-²H]-β-lactoside* **23**. To a solution of **20** (160 mg, 0.35 mmol) in dry CH_2Cl_2 (3 ml) was added silver trifluoromethanesulfonate (106 mg, 0.41 mmol), *N,N,N',N'*-tetramethylurea (50 µl, 0.41 mmol) and molecular sieves 3A (1.6 g). After being stirred under nitrogen for 30 min, the mixture was cooled (−78 °C) and a solution of tetra-*O*-acetyl-α-D-galactopyranosyl bromide[21] (170 mg, 0.41 mmol) in dry CH_2Cl_2 (3 ml) was added. The temperature was raised to 20 °C over 1 h and after an additional 4 h the reaction mixture was diluted with CH_2Cl_2 and filtered through Celite. The filtrate was washed with H_2O (15 ml) and saturated $NaHCO_3$ solution (15 ml), dried, concen-

trated and purified by preparative TLC by elution with ethyl acetate–hexane 1:2 to give (6*S*)-methyl 2,3,6-tri-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)[6-^{2}H]-β-D-glucopyranoside **22** (110 mg, 40 %) and starting material **20** (35 mg, 22 %). For NMR data see Tables 3 and 4.

A solution of **22** (110 mg, 0.14 mmol) in dry CH_3OH (10 ml) containing acetic anhydride (1 ml) and Pd–C (130 mg) was hydrogenated in an H_2 atmosphere overnight. The mixture was filtered through Celite and the residue was washed thoroughly with CH_3OH. The combined filtrate was concentrated (1 mmHg) and the product deacetylated overnight in CH_3OH (10 ml) containing 0.1 M $NaOCH_3$–CH_3OH (2 ml) to give crystalline **23** (31 mg, 63 %) which was filtered off and washed with cold CH_3OH. M.p. 208–210 °C, $[\alpha]_D^{25}$ +1.1° (*c* 0.8, water); lit.[22] (for non-deuteriated compound), m.p. 206 °C, $[\alpha]_D^{26}$ +5.6°. For NMR data see Tables 5 and 6.

Methyl β-allolactoside **25**. To a solution of methyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside[12] (300 mg, 0.65 mmol) in dry CH_2Cl_2 (5 ml) was added molecular sieves 3A, silver trifluoromethanesulfonate (200 mg, 0.78 mmol) and *N,N,N',N'*-tetramethylurea (93 µl, 0.78 mmol) and the mixture was stirred for 1 h under N_2. After cooling (−78 °C) a solution of tetra-*O*-acetylgalactopyranosyl bromide[21] (320 mg, 0.78 mmol) in dry CH_2Cl_2 (5 ml) was added and the mixture was stirred overnight (−23 °C). The mixture was heated to room temperature, filtered through Celite and charcoal, and purified by preparative TLC using ethyl acetate–hexane 1:1 as the eluent to give methyl 2,3,4-tri-*O*-benzyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **24** (205 mg, 40 %) which crystallized from ethanol. M.p. 151 °C, $[\alpha]_D^{25}$ +1.3° (*c* 0.9, $CHCl_3$). For NMR data see Tables 3 and 4. Anal. $C_{42}H_{50}O_{15}$: C, H.

In addition methyl 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside **26** (160 mg, 49 %) was isolated as a syrup, and identified by NMR spectroscopy (Tables 3 and 4).

Compound **24** was dissolved in ethyl acetate (10 ml), absolute ethanol (10 ml) and acetic acid (2 ml) and hydrogenated overnight using Pd–C (100 mg) as the catalyst. The mixture was filtered through Celite and concentrated and the product was deacetylated with $NaOCH_3$ in dry CH_3OH. After neutralization with Amberlite IR-120 methyl β-allolactoside **25** (44 mg, 51 %) was crystallized from ethanol. M.p. 133–135 °C, $[\alpha]_D^{25}$ −19.3° (*c* 1.1, water). For NMR data see Tables 5 and 6.

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) in 0.1 M sodium phosphate buffer (930 µl) then adding 30 mM dithiothreitol in D_2O 035 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), which gives pD = 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 to 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 means of the program REGGRAFA[15] in order to determine the rate constants V_0 and $t_{1/2}$.

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