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Derivatives of methyl β-lactoside as substrates for and inhibitors of β-D-galactosidase from *E. coli*

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ABSTRACT

The 2′-, 4′-, and 6′-deoxy derivatives of methyl β-lactoside have been synthesised by deoxygenation at positions 2′, 4′, and 6′, and the 3′-deoxy derivative was obtained by a glycosylation reaction. The 2′-*O*-methyl, 2′-*O*-benzyl, 2′-amino-2′-deoxy, and 1′-deuterio derivatives have been synthesized also. Only the 6′-deoxy and 1′-deuterio derivatives were substrates for the β-D-galactosidase from *E. coli*, and the 2′-deoxy- and 2′-amino-2′-deoxy derivatives were potent inhibitors for the hydrolysis of methyl β-lactoside by the enzyme.

INTRODUCTION

Many complex carbohydrate structures in biological systems play a vital role in recognition processes, *e.g.*, the blood-group determinants[1] and cell-surface carbohydrates that function as tumor-associated antigens and are recognized by monoclonal antibodies[2–5]. Furthermore, plant lectins bind specifically to cell-surface carbohydrates or oligosaccharides and may act as hormones in plant regulatory mechanisms[6].

The study of the interactions of carbohydrates and proteins has been facilitated by developments in high-field n.m.r. spectroscopy and f.a.b.-mass spectroscopy, by the availability of monoclonal antibodies[2], and by high-resolution X-ray structure analysis[7,8]. Studies of the binding of oligosaccharides, *e.g.*, those[9] related to the Lewis-b blood-group determinants, to lectins and antibodies have led Lemieux and co-workers to propose the hydrated polar-gate effect[10] on the strength and specificity of binding of oligosaccharides by proteins. Similar results have been found in studies of antibodies, transferases, fimbriae, or enzymes[11–14]. The latter results have been based on an appreciation of the preferred conformation of the oligosaccharides in solution, as determined by high-resolution n.m.r. spectroscopy and simple computer calculations in which the importance of the exo-anomeric effect was well appreciated (HSEA calculations)[15,16].

We have investigated carbohydrate–protein interactions by a study of the substrate specificity of the enzyme β-D-galactosidase from *E. coli* (EC 3.2.1.23), for which lactose is the natural substrate, by a systematic study of chemically modified substrate analogs of methyl β-lactoside[17]. Although the mechanism of hydrolysis by this enzyme

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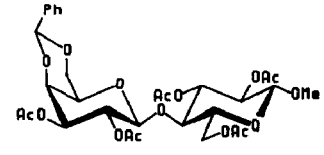
has been studied intensively[18,19], it has been used mainly for analytical purposes. Furthermore, most of the studies involved β-D-galactosides as substrates or substrate analogs.

We now describe the synthesis of a series of deoxy derivatives of lactose and also the 2′-*O*-methyl, or 2′-*O*-benzyl, and 2′-amino-2′-deoxy derivatives. The behaviour of these compounds as substrates or inhibitors[20] of the above β-D-galactosidase is reported also.

RESULTS AND DISCUSSION

The syntheses of the following compounds were not optimized because the main objective of the work was the enzymic study. The structure and purity of the unprotected derivatives and key intermediates were assessed by ^{1}H- and ^{13}C-N.m.r. spectroscopy.

Methyl 6′-deoxy-β-lactoside (**3**) was prepared from methyl 2,2′,3,3′,6-penta-*O*-acetyl-4′,6′-*O*-benzylidene-β-lactoside[21] (**1**) by opening of the 4′,6′-benzylidene ring with *N*-bromosuccinimide in tetrachloromethane[22] to give methyl 2,2′,3,3′,6-penta-*O*-acetyl-4′-*O*-benzoyl-6′-bromo-6′-deoxy-β-lactoside (**2**, 91%). Reduction of **2** with $NaBH_4$–$NiCl_2 \cdot 6H_2O$[23] then gave **3** (70% overall yield).



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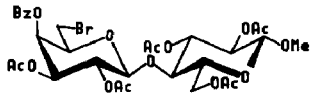

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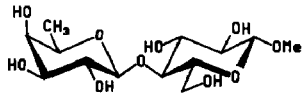

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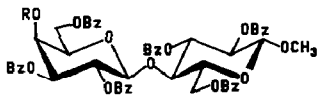
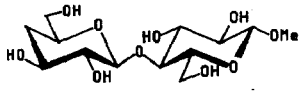


4 R = H

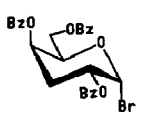
5 R = PhOCS



6

For the synthesis of methyl 4′-deoxy-β-lactoside (**6**), **4** (ref. 24) was treated with phenyl chlorothionocarbonate in dichloromethane[25], but only 2–3% of the acylated compound **5** was obtained. However, 75% could be obtained if the reaction was carried out in acetonitrile with 4-dimethylaminopyridine as the catalyst. Reduction of **5** with tributyltin hydride in toluene and deprotection gave **6**.

Methyl 3′-deoxy-β-lactoside (**10**) was synthesised from 3-deoxy-D-*xylo*-hexose[26], which was treated with benzoyl chloride in cold pyridine followed by reaction with hydrogen bromide in acetic acid to give mainly 2,4,6-tri-*O*-benzoyl-3-deoxy-α-D-*xylo*-hexopyranosyl bromide (**7**). The coupling of **7** to methyl 2,3,6-tri-*O*-benzyl-β-D-gluco-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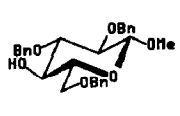
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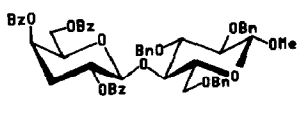
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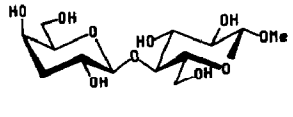
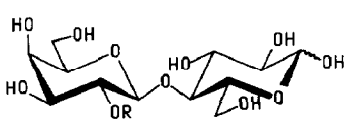

10

pyranoside[27] (**8**) was performed in dichloromethane and promoted by silver triflate and tetramethylurea to give **9** (50%), which was deprotected to give **10** (91%).

2′-*O*-Benzyl-lactose (**11**) and 2′-*O*-methyl-lactose (**17**) are available readily from 2,3:5,6:3′,4′-tri-*O*-isopropylidene-6′-*O*-triphenylmethyl-lactose dimethyl acetal[28] and were used for the synthesis of methyl 2′-deoxy-β-lactoside (**15**), methyl 2′-*O*-benzyl-β-lactoside (**16**), and methyl 2′-*O*-methyl-β-lactoside (**18**). Compound **11** was acetylated with acetic anhydride in pyridine and then treated with $TiBr_4$ in chloroform to give the glycosyl bromide. However, even under these mild reaction conditions, some debenzylation occurred. The mixture of products was glycosylated with methanol, promoted by silver carbonate, to give methyl 2,3,3′,4′,6,6′-hexa-*O*-acetyl-β-lactoside (**12**, 44%) and methyl 2,3,3′,4′,6,6′-hexa-*O*-acetyl-2′-*O*-benzyl-β-lactoside (**13**, 21%). Compound **13** was deacetylated to give **16**. Compound **12** was acylated with phenyl chlorothionocarbonate and 4-dimethylaminopyridine in acetonitrile to give a three-component mixture which could not be resolved easily by preparative t.l.c. The mixture was reduced with tributyltin hydride in toluene, and the products were purified by chromatography. The resulting methyl hexa-*O*-acetyl-2′-deoxy-β-lactoside (**14**) was *O*-deacetylated with methanolic sodium methoxide followed by reaction with solid CO_2 in order to avoid hydrolysis of the labile glycosidic linkage and yield **15**. When the above work had been finished, alternative and easier syntheses were reported[29,30]. Compound **17** (see ref. 28) was transformed into methyl 2′-*O*-methyl-β-lactoside (**20**) by treatment, in sequence, with acetic anhydride in pyridine, hydrogen bromide in acetic acid, silver carbonate in methanol, and methanolic sodium methoxide.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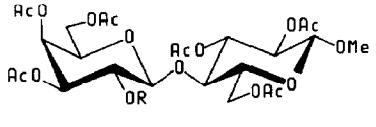
17 R = Me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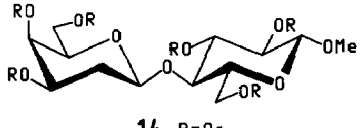
13 R = Bn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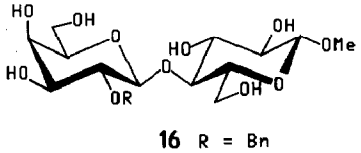
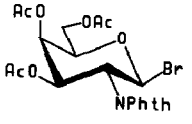
15 R=H


16 R = Bn

18 R = Me

The coupling of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl bromide[31,32] (**19**) with **8** in nitromethane was promoted by collidine and silver triflate to give methyl 3′,4′,6′-tri-*O*-acetyl-2,3,6-tri-*O*-benzyl-2′-deoxy-2′-phthalimido-β-lactoside (**20**, 39%), deprotection of which gave methyl 2′-amino-2′-deoxy-β-lactoside (**21**) isolated as the hydrochloride.

Methyl β-(1′-^{2}H)lactoside (**24**) was prepared by conversion of D-(1-^{2}H)galactose[33] penta-acetate into the α-glycosyl bromide **22**, coupling with **8**, and deprotection.



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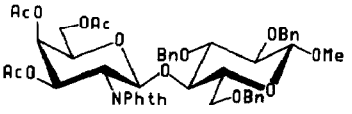

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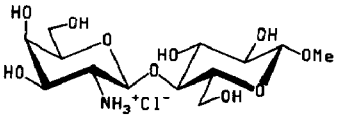

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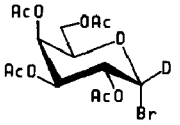

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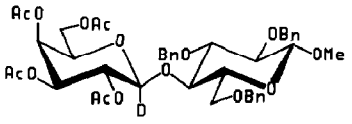

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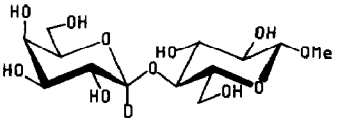

24

The above methyl β-lactoside derivatives were tested as substrates for the β-D-galactosidase from *E. coli*. Methyl β-lactoside was used as the reference compound rather than lactose in order to simplify n.m.r. analysis of the reaction mixtures. Only the β-(1′-^{2}H)lactoside **24** and 6′-deoxy-β-lactoside (**3**) derivatives were hydrolyzed by the enzyme, and the latter only slowly (Table I). On the other hand, many of the compounds inhibited the enzymic hydrolysis of methyl β-lactoside (Table I, Fig. 1). However, whereas the 2′-deoxy-β-lactoside **15** showed appreciable inhibitory properties, the 3′- (**10**) and 4′-deoxy (**6**) derivatives were weak inhibitors, and the 6′-deoxy derivative (**3**) was a non-inhibitor (Fig. 1, Table I). Thus, neither **10** nor **6** bind to the enzyme and therefore HO-3′ and HO-4′ probably function as essential polar gates[10] HO-6′ appears to be important for binding but is not essential since **3** is a substrate. These results accord with results on galactosides[34]. Since the 2′-deoxy derivative **15** is an inhibitor but not a substrate, HO-2′ is probably involved in the enzymic reaction. The importance of HO-2′ prompted an investigation of other 2′-derivatives. The 2′-*O*-methyl derivative (**18**) was not a substrate or an inhibitor, probably because MeO-2′ is too large to fit into the active site. The 2′-amino-2′-deoxy derivative **21**, which is mostly protonated under the reaction conditions, is not a substrate but is a good inhibitor.

Since glutamic acid-461 is present close to where the glycosidic linkage would be in the active site[35], it is believed that the inhibition is caused by an ionic bond between the carboxylate of the enzyme and the protonated amine of the substrate analogue. These results suggest a mechanism for the enzymic hydrolysis which explains the importance of HO-2′, namely, that it forms a hydrogen bond to the glutamic acid and forces it into a position close to the anomeric centre, where it would stabilize a galactosyloxonium ion

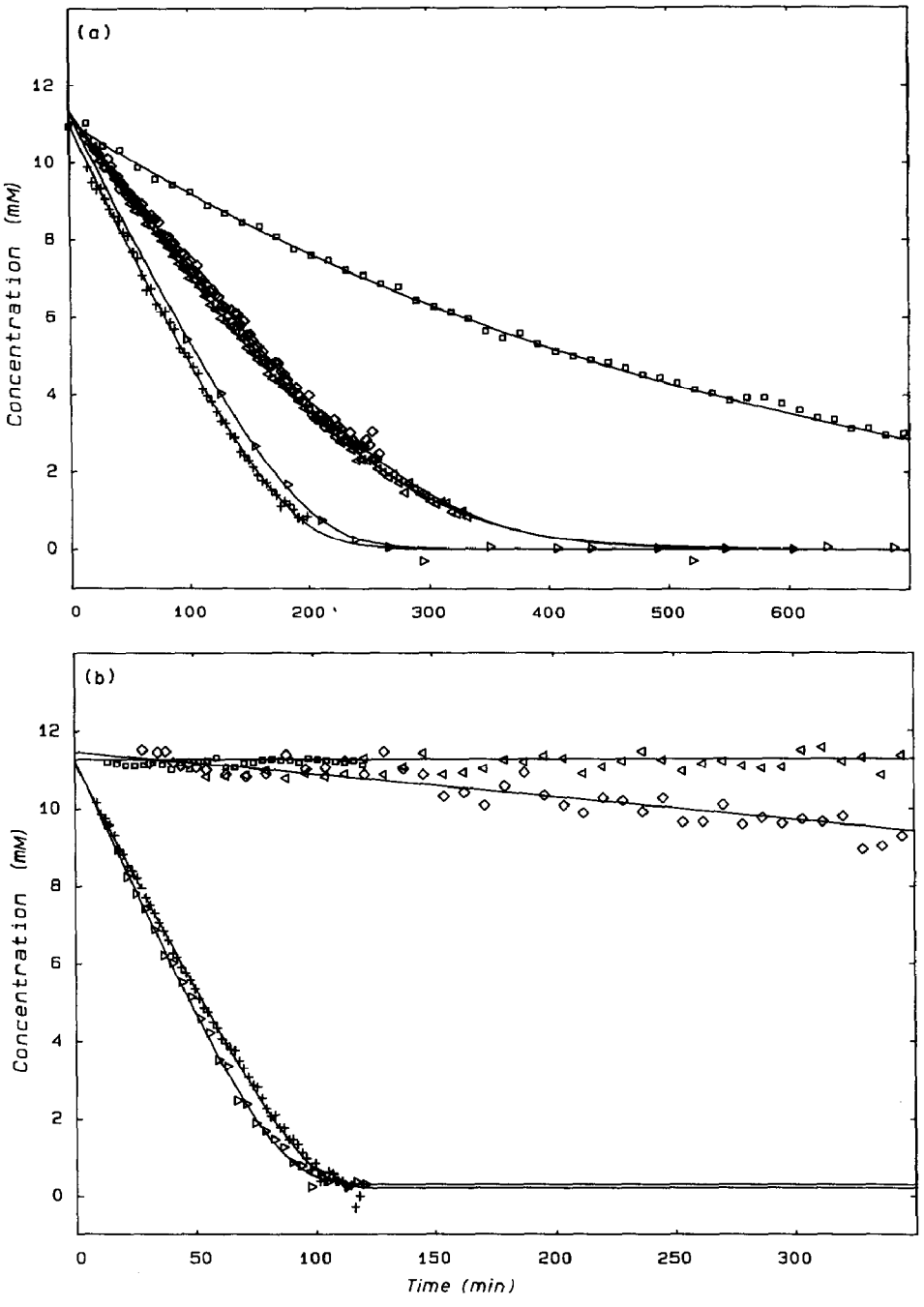

Fig. 1. Enzymic hydrolysis of (*a*) methyl β-lactoside alone (+) and in the presence of an equimolar amount of methyl 6′-deoxy-β-lactoside (▷), methyl 3′-deoxy-β-lactoside (△), methyl 4′-deoxy-β-lactoside (◇), and methyl 2′-deoxy-β-lactoside (□); and (*b*) methyl β-lactoside in competition with methyl 2′-*O*-methyl-β-lactoside (▷), methyl 2′-*O*-methyl-β-lactoside (□), methyl 2′-amino-2′-deoxy-β-lactoside (◇), and methyl 2′-amino-2′-deoxy-β-lactoside (◁).

TABLE I[a]

Kinetic parameters for enzymic hydrolyses

Single substrate experiments

Compound	V_0 *(mmol/min)*	$T_{\frac{1}{2}}$ *(min)*	K_m *(mM)*	V_{max} *(mmol/min)*
Methyl β-lactoside[b]	0.065	88.2	1.57	0.075
6′-deoxy (**3**)[b]	0.003	2723	109962	27.995
Methyl β-lactoside[c]	0.111	52.2	0.47	0.115
Methyl β-lactoside[c]	0.112	51.7	0.49	0.116
1′-^{2}H (**24**)[c]	0.115	50.3	0.64	0.122
1′-^{2}H (**24**)[c]	0.113	50.6	1.11	0.124

Competition experiments[d]

Compound	V_0 *(mmol/min)*	$T_{\frac{1}{2}}$ *(min)*
6′-Deoxy (**3**)[b]	0.063	89.4
4′-Deoxy (**6**)[b]	0.044	147.8
3′-Deoxy (**10**)[b]	0.038	136.4
2′-Deoxy (**15**)[b]	0.020	399.8
2′-OMe (**18**)[c]	0.110	51.9
2′-Amino-2-deoxy (**21**)[c]	0.006	1018

[a] The accuracy of the data are: $V_0 < 1\%$, $T_{\frac{1}{2}} < 1\%$, $K_m > 100\%$[20], and $V_{max} < 2\%$. [b] The enzyme concentration was ~24nM. [c] The enzyme concentration was ~38nM. [d] Methyl β-lactoside in the presence of an equimolar amount of an inhibitor.

or form a covalently linked galactosyl intermediate. If HO-2′ is absent (as in **15**), then the stabilization of the intermediate cannot occur and hydrolysis will not be promoted. This mechanism is visualised in Fig. 2.

Methyl β-(1′-^{2}H)lactoside was tested as substrate but there was no significant deuterium effect (Table I), which accords with the results of Sinnott *et al.*[35], who showed that substrates that were hydrolysed slowly by the enzyme showed no deuterium effect on the rate of hydrolysis.

EXPERIMENTAL

General procedures. — ^{1}H-N.m.r. spectra were recorded with Bruker AM-500 and Bruker AC-250 instruments at 27° for solutions in $CDCl_3$ (internal Me_4Si). The HDO peak was used as the internal reference (4.75 p.p.m.) for solutions in D_2O. ^{13}C-N.m.r. spectra were recorded with Bruker AM-500, AC-250, and WH-90 spectrometers. $CDCl_3$ was used as internal reference (76.9 p.p.m.) for solutions in $CDCl_3$, and an external instrument reference was used for solutions in D_2O (1,4-dioxane, 67.4 p.p.m.). The n.m.r. data are given in Tables II–V. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. T.l.c. was performed on Silica Gel 60 F_{254} (Merck). After preparative t.l.c., the products were extracted with ethyl acetate. All reactions in organic

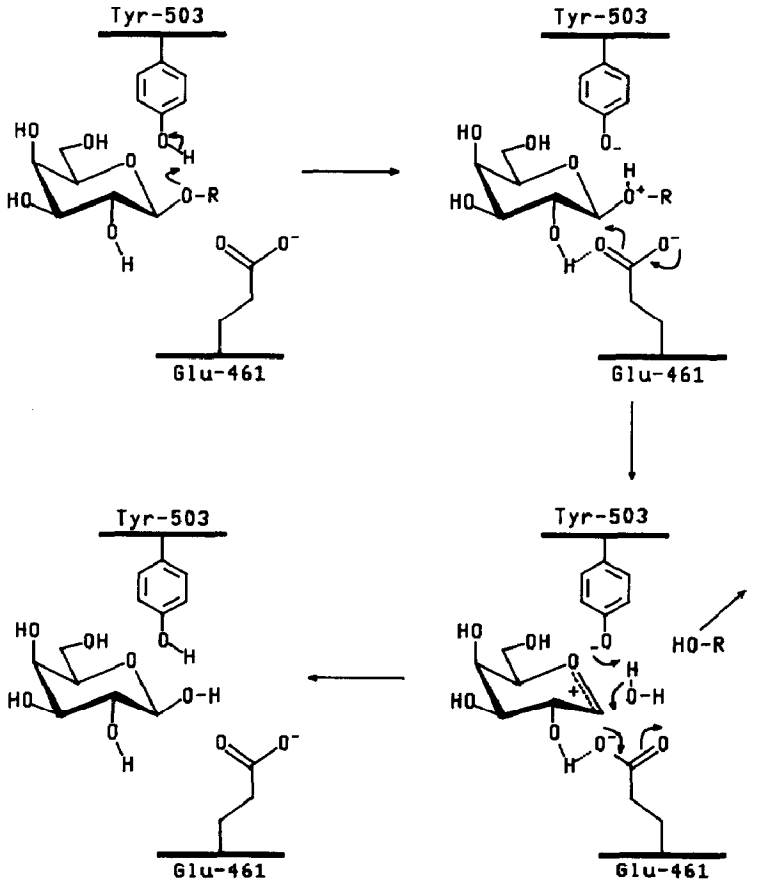

Fig. 2. Proposed mechanism for the enzymic hydrolysis of lactose by β-D-galactosidase from *E. coli*. The mechanism explains the essential function of HO-2'.

solvents were carried out with the exclusion of moisture, and solvents for critical reactions were dried over molecular sieves. Concentrations were carried out under diminished pressure at $\leq 50°$, unless otherwise stated. Melting points are uncorrected. Elemental analysis was performed by Løven A/S's microanalytical laboratory.

Methyl 2,3,6-tri-O-acetyl-4-O-(2,3-di-O-acetyl-4-O-benzoyl-6-bromo-6-deoxy-β-D-galactopyranosyl)-β-D-glucopyranoside (**2**). — Methyl 2,3,6-tri-*O*-acetyl-4-*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-β-D-glucopyranoside[21] (**1**; 1.08 g, 1.7 mmol) was dissolved in CCl_4 (100 mL) and half of the solvent was evaporated in order to remove traces of water. Anhydrous $BaCO_3$ (3 g) and *N*-bromosuccinimide (285 mg) were added, the mixture was boiled until the bromine color had disappeared, the inorganic salts were collected and washed with CH_2Cl_2, and the combined filtrate and washings were concentrated to dryness. A solution of the residue in CH_2Cl_2 (60 mL) was washed with water (3 × 25 mL), dried ($MgSO_4$), and concentrated, and the residue was crystallized from CH_2Cl_2–pentane (1:20) to give **2** (1.1 g, 91%), m.p. 99–104°, $[\alpha]_D^{25}$ + 15° (*c* 1.3, chloroform).

TABLE II

^{1}H-N.m.r. data (δ in p.p.m., J in Hz)[a]

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*	*H-1'*	*H-2'*	*H-3'*	*H-4'*	*H-5'*	*H-6'a*	*H-6'b*	*OMe*
3	4.37 d 8.0	3.27 m 9.0	[b] m –	[b] m –	[b] m 4.8, 1.8	3.80 dd 12.3	3.95 dd	4.36 d 7.8	3.46 dd 10.0	3.62 dd 3.2	3.72 dd 0.9	3.83 dqv 6.4	1.23 d		3.57
8	4.45 d 8.1	3.36 dd 9.0	3.66 t 9.2	3.81 t 9.7	3.66 m 1.7, 4.7	4.05 dd 12.0	3.87 dd	4.48 d 7.9	3.27 dd 9.3	3.60 m 12.0	1.50 q 12.0 2.03 ddd 5.1, 1.9	2.67 m 6.1, 6.0	3.80 dd 11.4 $J_{4',4'}$ = 13.0	3.84 dd	3.63
10	4.41 d 8.1	3.32 dd 9.6	3.65 m –	3.76 m –	3.61 m 2.0, 5.2	3.99 dd 12.1	3.81 dd	4.45 d 8.0	3.75 m 12.2	1.75 m 2.9 2.22 m 5.6, 3.1	4.00 bs – $J_{3',3'}$ = 13.9	3.66 m –	3.72–3.79 m –	– –	3.58
15	4.41 d 8.0	3.32 dd 9.1	3.63 t 9.0	3.69 t 9.4	3.56 ddd 2.2, 5.1	3.92 dd 12.3	3.75 dd	4.71 dd 2.0	2.09 m 4.8 1.71 m 9.9, 12.9	3.90 ddd 3.1 $J_{2',2'}$ = 12.1	3.79 bd 1.0	3.62 ddd 4.1, 8.1	3.82 dd 11.9	3.77 dd	3.58

16	4.27	3.20	3.40——3.68			3.68	3.77	4.37	3.46	3.60	3.82	——	3.40–3.68 ——		3.47
	d	m	m	m	m	dd	dd	d	dd	dd	bd	m	m	m	
	7.9	9.4	–	–	3.6, 2.0	12.0		7.9	9.8	3.2	0.4	–	–	–	
18	4.41	3.27	3.76-3.82		3.64–3.70	3.75	3.99	4.49	3.32	3.68	3.92		— 3.64–3.70 ——		3.58, 3.60
	d	dd	m	m	m	dd	dd	d	dd	dd	bd	m	m	m	
	8.1	9.9	–	–	4.0, 0.8	12		7.9	10.0	3.4	–	–	–	–	
21	4.41	3.32	3.70	3.82	3.66	3.76	3.92	4.81	3.27	3.91	3.98	3.78	3.77–3.80	3.83	3.75
	d	dd	t	t	ddd	dd	dd	d	dd	dd	bd	m	m	dd	
	8.0	9.4	9.2	9.9	4.8, 2.8	12.5		8.4	11.0	3.4	0.8	–, 8.6	12.2		
24	4.40	3.30	3.58–3.69		3.60	3.98	3.80	–	3.53	3.66	3.91	——	3.70–3.78 ——		3.56
	d	m	m	m	m	dd	dd	–	d	dd	bd	m	m	m	
	7.9	9.5	–	–	1.9, 4.6	12.0			9.5	3.2	0.5	–	–	–	

[a] Measured at 500 MHz at 27° in D_2O, using HDO as internal reference at 4.75 p.p.m. Coupling constants, given below the chemical shifts, were measured on a first-order basis. [b] The chemical shifts were not assigned.

TABLE III

^{13}C-N.m.r. data[a]

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	*OCH_3*	
3	104.2	74.1	75.7	80.2	76.0	61.4	104.3	71.9	74.0	72.3	72.5	16.6	58.5	
6	103.8	74.8	75.6	79.9	75.8	61.0	103.9	71.0	73.7	34.8	75.2	64.3	58.0	
10	104.5	74.3	75.9	80.1	76.2	61.6	106.4	67.0	38.5	67.1	79.8	62.7	58.7	
15	103.9	73.6	75.2	79.1	75.5	61.0	101.1	34.3	68.5	67.5	76.4	62.2	58.0	
16	103.9	73.6	75.6	80.3	75.0	60.7	103.0	78.1	73.3	69.6	75.1	61.9	58.0	OBn 76.4
18	103.8	73.6	75.7	79.1	76.0	60.7	103.4	81.7	73.0	69.3	75.1	61.4	58.0	OMe 61.8
21	103.1	73.1	74.0	76.6	74.4	60.6	97.9	53.7	69.2	67.7	75.9	61.0	57.4	
24	103.9	73.7	75.3	79.2	75.6	60.9	–	71.7	73.4	69.4	76.2	61.9	58.1	

[a] Measured at 125.7 MHz, at 27° in D_2O, using 1,4-dioxane as external reference as 67.4 p.p.m.

Anal. Calc. for $C_{30}H_{37}BrO_{16}$: C, 49.12; H, 5.08; Br, 10.89. Found: C, 49.17; H, 5.16; Br, 10.80.

Methyl 6'-deoxy-β-lactoside (**3**). — To a solution of **2** (603 mg, 0.8 mmol) in ethanol (35 mL) at 0° were added $NiCl_2 \cdot 6H_2O$ (655 mg, 2.8 mmol) and $NaBH_4$ (815 mg, 21 mmol). The mixture was boiled under reflux for 1 h, then cooled, filtered through Celite and charcoal, neutralized with Amberlite IRC-50 (H^+) resin, and concentrated. Elution of the residue from a column of Sephadex G-15 with CH_3OH–H_2O (1:1) gave **3** (216 mg, 77%), m.p. 234–235° (from EtOH), $[\alpha]_D^{25}$ −9° (*c* 1.1, deuterium oxide). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{13}H_{24}O_{10}$: C, 45.88; H, 7.11. Found: C, 45.33; H, 7.10.

Methyl 4'-deoxy-β-lactoside (**6**). — To a solution of $\mathbf{4}^{24}$ (312 mg, 0.32 mmol) and 4-dimethylaminopyridine (194 mg, 5 equiv.) in dry CH_3CN (5 mL) under N_2 was added phenyl chlorothionocarbonate (70 μL, 1.5 equiv.). The mixture was stirred for 42 h and concentrated, and the residue was partitioned between EtOAc (15 mL) + H_2O (15 mL). The organic phase was washed with M HCl (2 × 10 mL) and saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and concentrated. Preparative t.l.c. (EtOAc–hexane, 1:2; 3 irrigations) gave methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,6-tri-*O*-benzoyl-4-*O*-phenoxythiocarbonyl-β-D-galactopyranosyl)-β-D-glucopyranoside (**5**; 250 mg, 71%) as the main fraction, which was identified by the ^{1}H-n.m.r. data (Table IV).

To a solution of **5** (250 mg, 0.22 mmol) in dry toluene (5 mL) was added *α,α'*-azobisisobutyronitrile (16 mg) and Bu_3SnH (90 μL, 0.34 mmol) under N_2. The mixture was heated to 80° for 4 h, then cooled to room temperature, and concentrated. Preparative t.l.c. (EtOAc–hexane, 1:2) of the residue gave a fraction (242 mg) that was crystallized from ethanol to give a product (195 mg) whose ^{1}H-n.m.r. spectrum (500 MHz) indicated the presence of 80–90% of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,6-tri-*O*-benzoyl-4-deoxy-β-D-*xylo*-hexopyranosyl)-β-D-glucopyranoside (See Table IV). A solution of this product in 0.1M NaOMe/MeOH (5 mL) was stirred for 18 h at 30–35°, then neutralized with mixed-bed MB3 resin, and concentrated. A solution of the residue in H_2O (15 mL) was washed with hexane (2 × 7 mL), then lyophilized to give **6** (31 mg). Recrystallisation from ethanol gave a hygroscopic solid (12 mg), m.p. 183–85°, $[\alpha]_D^{25}$ −14° (*c* 1.2, water). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{13}H_{24}O_{10} \cdot 0.5H_2O$: C, 44.70; H, 7.21. Found: C, 44.66; H, 6.89.

Methyl 4-O-(3-deoxy-β-D-xylo-hexopyranosyl)-β-D-glucopyranoside (**10**). — To a solution of 3-deoxy-D-*xylo*-hexose[26] (604 mg, 3.7 mmol) in pyridine (10 mL) at −5° was added benzoyl chloride (5 mL) in pyridine (15 mL) dropwise during 45 min. The mixture was stirred for 3.5 h at room temperature, then poured into ice-cold saturated aqueous $NaHCO_3$ (50 mL), and extracted with CH_2Cl_2 (75 mL). The extract was washed with 4M HCl (3 × 35 mL) and aqueous $NaHCO_3$ (3 × 25 mL), dried ($MgSO_4$), and concentrated. To a solution of the residue in CH_2Cl_2 (20 mL) at 0° was added saturated HBr–AcOH (2 mL). The mixture was stirred for 1.5 h, then diluted with CH_2Cl_2 (30 mL), washed with saturated aqueous $NaHCO_3$ (3 × 20 mL), dried ($MgSO_4$), and concentrated, and the residue was kept in the dark in a desiccator over KOH. T.l.c. (EtOAc–hexane, 1:2) revealed one main product with R_F 0.78. ^{1}H-N.m.r. (500 MHz)

TABLE IV

^{1}H-N.m.r. data of derivatives[a]

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*	*H-1'*	*H-2'*	*H-3'*	*H-4'*	*H-5'*	*H-6'a*	*H-6'b*	*OMe*
5	4.60	5.44	5.78	4.25	3.79–3.89	4.48	4.61	4.86	5.69	5.41	6.13	3.79–3.89	3.71	3.79–3.89	3.44
	d	dd	t	t	m	dd	dd	d	dd	dd	dd	m	dd	–	
	7.8	9.9	9.6	9.6	4.3, 1.8	12.1		8.0	10.3	3.6	0.6	6.0, –	11.6		
[b]	4.57	5.49	5.76	4.22	3.81	4.50	4.61	4.74	5.47	5.20	1.59	3.66	3.71	3.87	3.43
	d	dd	t	t	ddd	dd	dd	d	dd	m	q	m	dd	dd	
	7.9	9.8	9.4	9.5	4.3, 2.0	12.0		7.9	9.8	11.9	11.9	5.8, 4.2	11.5		
											2.24				
											dd				
											5.4, 1.6, $J_{4',4''}$ = 12.1				
7	–	–	–	–	–	–	–	6.95	5.37	2.44	5.65	4.67	4.48	4.58	
								d	dt	m	bs	m	dd	dd	
								3.6	4.2	2	–	5.7, 7.0	11.6		
										2.58					
										dd					
										11.8, 3.0, $J_{3',3''}$ = 14.0					
9	4.25	3.41	4.09	3.63	3.36	3.78	3.71	4.92	5.25	1.78	5.38	3.91	4.35	4.30	3.52
	d	dd	t	t	m	dd	dd	d	m	ddd	bs	t	dd	dd	
	7.9	9.2	9.6	9.1	3.8, 1.2	11.5		8.0	12.0	3.1	–	6.5, 6.9	11.0		
										2.61					
										ddd					
										5.1, 3.1, $J_{3',3''}$ = 14.0					

12	4.40 d 8	4.90 dd 9	5.22 t 9	3.84 t 9	3.68 ddd 5.2	4.32 dd 12	4.60 dd	4.43 d 8	3.71 dd 10	4.87 dd 3	5.33 dd 1	3.89 dt –	—4.05–4.13— m m – –		3.50
13	4.40 d 7.8	4.91 dd 9.6	5.22 t 9.5	3.83 t 9.2	3.57 m 1.2, 3.8	4.17 dd 12.0	4.51 dd	4.41 d 7.7	3.53 dd 10.3	4.93 dd 3.3	5.31 bd 0.8	3.85 m 7.2, 6.5	4.07 dd 11.4	4.11 dd	3.46
[c]	4.44 d 7	4.90 dd 10	5.26 t 10	3.91 t 10	3.68 ddd 4.2	4.28 dd 12	4.62 dd	4.73 d 7	5.70 dd 10	5.18 dd 3	5.43 dd 1	3.67 dt –	—4.08–4.20— m m – –		3.49
14	4.41 d 7.9	4.91 dd 9.8	5.20 t 9.6	3.87 t 9.6	3.64 ddd 4.5, 2.4	4.20 dd 12.0	4.42 dd	4.56 dd 9.2	1.88 ddd 11.9 2.00–2.20 m 5.2, –	4.94 ddd 3.2	5.22 bd 0.8	3.78 m –	—4.10— m –		3.49
19	–	–	–	–	–	–	–	6.40 d 9.3	4.85 dd 10.8	5.78 dd 3.2	5.55 d 0	5.30 s –	5.30 s –	5.30 s –	
20	4.18 d 7.9	3.37 dd 9.0	4.01 t 9.0	3.60 t 8.9	3.26 m 4.0, 1.1	3.40 dd 11.2	3.51 dd	5.65 d 8.1	4.49 dd 11.4	5.70 dd 3.2	5.34 bd 0.8	3.56 m 5.5, 8.2	3.80 dd 10.7	4.08 dd	3.46
23	4.28 d 7.6	3.40 dd 9.0	3.96 dd 9.5	3.57 t 9.0	3.36 m –	3.73 d –		–	4.87 d 10.8	4.80 dd 3.6	5.25 dd 1.3	3.51 m 8.1, 5.8	4.01 dd 12.4	3.84 dd	3.56

[a] Measured at 500.1 MHz and at 27° in $CDCl_3$ (internal Me_4Si). Coupling constants, given below the chemical shifts, were measured on a first-order basis. [b] Methyl 2,2′,3,3′,6,6′-hexa-*O*-benzoyl-4′-deoxy-β-lactoside. [c] Methyl 2,3,3′,4′,6,6′-hexa-*O*-acetyl-2′-*O*-phenoxythiocarbonyl-β-lactoside.

TABLE V

^{13}C-N.m.r. data of derivatives[a]

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	OCH_3		
14	101.3	71.2	72.4	74.8	72.5	62.1	99.4	31.8	67.9	64.7	70.9	61.3	56.7		
19	–	–	–	–	–	–	77.9	54.4	67.7	66.8	75.7	61.2	–		
20	104.3	82.6	81.8	75.2	74.1	67.7	97.4	52.0	67.8	66.2	70.4	60.5	56.7		74.9
23	104.5	81.5	82.3	76.2	73.4	69.4	99.8	67.6	70.2	66.6	70.8	60.4	56.6	OBn	74.5
															74.4

[a] Measured at 125.7 MHz and 27° in $CDCl_3$, using $CDCl_3$ as the internal reference at 76.9 p.p.m.

spectroscopy identified this product as the glucosyl bromide **7**. The n.m.r. data are given in Table IV.

A solution of methyl 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside[27] (**8**; 300 mg, 0.65 mmol) in dry CH_2Cl_2 (5 mL) that contained silver trifluoromethanesulfonate (200 mg, 0.78 mmol), molecular sieves 3 Å (1.7 g), and tetramethylurea (93 μL, 0.78 mmol) was stirred under N_2 for 30 min, then cooled to −78°. A solution of crude **7** (500 mg) in dry CH_2Cl_2 (5 mL) was added with the exclusion of moisture, and the mixture was stirred for 18 h at −23°, then diluted with CH_2Cl_2 (10 mL), and filtered through Celite. The filter pad was washed with CH_2Cl_2 (20 mL), and the combined filtrate and washings were washed successively with aqueous $NaHCO_3$ (15 mL), H_2O (15 mL), 4M HCl (10 mL), H_2O (10 mL), and aqueous $NaHCO_3$ (10 mL), dried ($MgSO_4$), and concentrated. Preparative t.l.c. (EtOAc–hexane, 1:2) gave two main fractions. The fastest running fraction had R_F 0.61 and was a 1:1 mixture (414 mg) of 2,3,6-tri-*O*-benzyl-4-*O*-(2,4,6-tri-*O*-benzoyl-3-deoxy-β-D-*xylo*-hexopyranosyl)-β-D-glucopyranoside (**9**) and (**8**). This mixture was acetylated conventionally with Ac_2O–pyridine, and the product was purified by preparative t.l.c. (EtOAc–hexane, 1:2) to give syrupy **9** (296 mg, 50% yield from **8**). The n.m.r. data are given in Table IV.

A solution of **9** (290 mg, 0.32 mmol) in CH_2Cl_2 (0.5 mL) and 0.05M NaOMe–MeOH (20 mL) was stirred overnight with the exclusion of moisture, then neutralized with Amberlite IR-120 (H^+) resin and concentrated. A solution of the residue in MeOH (21 mL) and AcOH (4 mL) was hydrogenated in H_2 over 10% Pd–C (57 mg) for 19 h, then filtered through Celite, and the filter pad was washed with MeOH–H_2O (1:1). The combined filtrate and washings were concentrated at 1 mmHg and the residue was eluted from a column of Sephadex G-15 with MeOH–H_2O (1:1). Lyophilization gave **10** (101 mg, 94%) as a hygroscopic solid, $[\alpha]_D^{25}$ −31° (*c* 1.3, deuterium oxide). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{13}H_{24}O_{10}\cdot H_2O$: C, 43.57; H, 7.31. Found: C, 43.69; H, 7.15.

*Methyl 2,3,6-tri-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside* (**12**) *and methyl 2,3,6-tri-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside* (**13**). — Treatment of 2′-*O*-benzyl-lactose[28] (**11**; 804 mg, 1.86 mmol) with Ac_2O (16 mL) and pyridine (20 mL) for 20 h followed by conventional work-up gave pure (t.l.c.) syrupy 1,2,3,6-tetra-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-*O*-benzyl-β-D-galactopyranosyl)-D-glucopyranose. This hepta-acetate (1.20 g, 1.65 mmol) was dissolved in dry $CHCl_3$ (65 mL) that had been freshly filtered through basic Al_2O_3 to remove ethanol, titanium tetrabromide (2.0 g, 5.5 mmol) was added, and the mixture was boiled for 18 h under reflux under N_2. The cooled mixture was washed with water (4 × 20 mL) and saturated aqueous $NaHCO_3$ (3 × 20 mL), then concentrated. To a solution of the residue in CH_2Cl_2 (20 mL) was added dry MeOH (20 mL) and Ag_2CO_3 (1.63 g, 5.9 mmol). The mixture was stirred in the dark for 20 h, then filtered through charcoal and Celite, and concentrated. Preparative t.l.c. (EtOAc–hexane, 3:1) of the residue gave, as the slowest moving fraction, **12** (441 mg, 44%) and, as the fastest fraction, **13** (238 mg, 21%), which were identified by ^{1}H-n.m.r. spectroscopy and not purified further. The n.m.r. data are given in Table IV.

Methyl 2'-deoxy-β-lactoside (**15**). — To a solution of **12** (422 mg, 0.69 mmol) and 4-dimethylaminopyridine (424 mg, 5 equiv.) in dry CH_3CN (10 mL) under N_2 was added phenyl chlorothionocarbonate (144 μL, 1.5 equiv.). After stirring the mixture for 22 h, t.l.c. (EtOAc–hexane, 1:1) showed ~75% reaction. More chlorothionocarbonate (50 μL, 0.5 equiv.) was added, the mixture was stirred for 6.5 h and then concentrated, and the residue was partitioned between EtOAc (35 mL) and H_2O (15 mL). The aqueous phase was extracted with EtOAc (10 mL), and the combined organic extracts were dried (Na_2SO_4) and concentrated. Preparative t.l.c. (EtOAc–hexane, 1:1) of the mixture gave a main fraction (271 mg) that contained 3 compounds in the molar ratios 1:2:6. The main component was identified by ^{1}H-n.m.r. spectroscopy (see Table IV) as methyl 2,3,3',4',6,6'-hexa-*O*-acetyl-2-*O*-phenoxythiocarbonyl-β-lactoside, and **12** (121 mg, 29%) was recovered. To a solution of the crude thiocarbonate (270 mg) and *a,a'*-azobisisobutyronitrile (21 mg) in dry toluene (10 mL) under N_2 was added Bu_3SnH (110 μL, 1.1 equiv.), and the mixture was stirred for 4 h at 80°. Concentration and preparative t.l.c. (EtOAc–hexane, 2:1) yielded a syrup (147 mg, R_F 0.64), which crystallized from ether–hexane to give **14** (124 mg, 86%), m.p. 121–123°, $[\alpha]_D^{25}$ −7.4° (*c* 0.5, chloroform). The n.m.r. data are given in Tables IV and V.

Anal. Calc. for $C_{25}H_{36}O_{16}$: C, 50.67; H, 6.12. Found: C, 50.86; H, 6.19.

Compound **14** (72 mg) was deacetylated in dry MeOH (10 mL) + 0.1M NaOMe–MeOH (0.4 mL) by stirring overnight at room temperature. Neutralization with solid CO_2 and elution of the product from a column of Sephadex G-15 with MeOH–H_2O (1:1) yielded **15** (28 mg, 67%), m.p. 214–216°, $[\alpha]_D^{25}$ −2.4° (*c* 0.2, water). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.74; H, 6.94. Found: C, 51.73; H, 6.93.

Methyl 2'-O-benzyl-β-lactoside (**16**). — Compound **13** (238 mg, 0.34 mmol) was *O*-deacetylated with 0.1M NaOMe–MeOH (2 mL) in dry MeOH (15 mL), to give **16** (95 mg, 62%) which crystallized from the reaction mixture. Recrystallization from ethanol gave a hygroscopic solid, m.p. 240–243°, $[\alpha]_D^{25}$ −7.1° (*c* 1.1, water). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.74; H, 6.94. Found: C, 51.73; H, 6.93.

Methyl 2'-O-methyl-β-lactoside (**18**). — 2'-*O*-Methyl-lactose[28] (**17**; 226 mg, 0.63 mmol) was acetylated with Ac_2O (10 mL) in pyridine (20 mL) for 16 h. The mixture was diluted with CH_2Cl_2 (40 mL), cooled to 0°, and washed with cold 4M HCl (3 × 20 mL) and saturated aqueous $NaHCO_3$ (2 × 20 mL), dried ($MgSO_4$), and concentrated. To a solution of the resulting hepta-acetate in CH_2Cl_2 (15 mL) was added saturated HBr–AcOH (5 mL). After stirring for 50 min, the mixture was diluted with CH_2Cl_2 (20 mL) and washed with ice–water (2 × 20 mL) and saturated aqueous $NaHCO_3$ (2 × 20 mL), dried ($MgSO_4$), and concentrated. To a solution of the resulting glycosyl bromide in CH_2Cl_2 (10 mL) were added dry MeOH (10 mL) and Ag_2CO_3 (550 mg, 2.0 mmol), and the mixture was stirred overnight in the dark, then filtered through charcoal and Celite, and concentrated to give crude syrupy methyl 2,3,3',4',6,6'-hexa-*O*-acetyl-2'-*O*-methyl-β-lactoside (341 mg), which was deacetylated in dry MeOH (20 mL) and 0.1M NaOMe–MeOH (1.2 mL). The mixture was neutralized with Amberlite IR-120 (H^+) resin, and

the residue was crystallized from ethanol to give **18** (70 mg, 28% overall yield), as a hygroscopic solid, m.p. 172–173°, $[\alpha]_D^{25}$ +7.7° (*c* 0.9, water). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{14}H_{26}O_{11}\cdot 0.5H_2O$: C, 44.33; H, 7.17. Found: C, 43.76; H, 7.02.

Methyl 2'-amino-2'-deoxy-β-lactoside (**21**). — To a solution of **8** (ref. 27) in CH_3NO_2, were added 2,4,6-trimethylpyridine (80 μL, 0.6 mmol) and molecular sieves (3Å), and the mixture was stirred under N_2. After 1 h, silver triflate (156 mg, 0.6 mmol) was added, the temperature was lowered to −40°, and a solution of **19** (ref. 32) (215 mg, 0.45 mmol) in CH_3NO_2 (5 mL) was added dropwise during 1 h. The mixture solidified and, after 3 h, was melted by raising the temperature to 20–22°. After an additional 1.3 h, the mixture was diluted with $CHCl_3$ (10 mL), and filtered through Celite, and the filter pad was washed with $CHCl_3$ (10 mL). The combined filtrate and washings were washed successively with M HCl (2 × 10 mL) and saturated aqueous $NaHCO_3$ (10 mL), dried ($MgSO_4$), and concentrated. Preparative t.l.c. (EtOAc–hexane, 1:1) of the residue gave methyl 2,3,6-tri-*O*-benzyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-β-D-glucopyranoside (**20**; 159 mg, 39%). The product was identified by n.m.r. spectroscopy (Tables IV and V). Deacetylation of **20** (150 mg) with NH_3–MeOH overnight gave, quantitatively, methyl 2,3,6-tri-*O*-benzyl-4-*O*-(2-deoxy-2-phthalimido-β-D-galactopyranosyl)-β-D-glucopyranoside, which was boiled with 80% hydrazine hydrate (2 mL) in 95% ethanol (20 mL) to deprotect the amino function and then hydrogenated overnight over 10% Pd–C in methanol (25 mL) containing 2 drops of conc. HCl. The product was eluted from a column of Sephadex G-15 with MeOH–water (1:1) to give **21**, isolated as a syrup (41 mg, 60%), $[\alpha]_D^{25}$ −2.1° (*c* 2.3, deuterium oxide). The n.m.r. data are given in Tables II and III.

Methyl 4-O-β-D-(1-^{2}H)galactopyranosyl-β-D-glucopyranoside (**24**). — To a solution of β-D-(1-^{2}H)galactopyranose penta-acetate[33] (250 mg, 0.63 mmol) in CH_2Cl_2 (10 mL) at 0° was added saturated HBr–AcOH (5 mL). The mixture was stirred for 1 h at room temperature, then diluted with CH_2Cl_2 (25 mL), washed with ice–water (2 × 20 mL) and aqueous $NaHCO_3$ (2 × 15 mL), dried ($MgSO_4$), and concentrated to give crude crystalline 2,3,4,6-tetra-*O*-acetyl-α-D-(1-^{2}H)galactopyranosyl bromide (**22**; 260 mg, 0.63 mmol).

A solution of **8** (ref. 27) (330 mg, 0.71 mmol), silver trifluoromethanesulfonate (161 mg, 0.63 mmol), and tetramethylurea (78 μL, 0.65 mmol) in dry CH_2Cl_2 (10 mL) over molecular sieve (3Å) was stirred for 1 h under N_2, then cooled to −15°. A solution of **22** (260 mg, 0.63 mmol) in dry CH_2Cl_2 (4 mL) was added, the temperature was raised slowly to room temperature, and stirring was continued for 21 h. T.l.c. (EtOAc–hexane, 1:1) then showed almost complete reaction. The mixture was filtered and concentrated at <30°. Preparative t.l.c. (EtOAc–hexane, 1:1) of the residue gave, as the main fraction, methyl 2,3,6-tri-*O*-benzyl-4-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-(1-^{2}H)galactopyranosyl]-β-D-glucopyranoside (**23**; 244 mg, 48% yield), isolated as a syrup. The n.m.r. data are given in Tables IV and V. The fastest running fraction was **8** (190 mg, 0.41 mmol).

A solution of **23** (240 mg, 0.30 mmol) in EtOAc (10 mL), EtOH (10 mL), and AcOH (2 mL) was hydrogenated overnight over 10% Pd–C (100 mg), then filtered

through Celite, and concentrated at 1mmHg. The product was deacetylated with 0.1M NaOMe–MeOH (2 mL) in MeOH (10 mL) overnight. The solution was neutralized with Amberlite IR-120 (H^+) resin and concentrated, and the resulting syrup (80 mg) was crystallized from EtOH to give **24** (57 mg, 53%), m.p. 210°, $[\alpha]_D^{25}$ +0.9° (*c* 1.4, water). The n.m.r. data are given in Tables II and III.

Anal. Calc. for $C_{13}H_{23}DO_{11}\cdot 1.5H_2O$: C, 40.58; H, 7.28. Found: C, 40.36; H, 7.07.

Enzymic procedures. —β-D-Galactosidase (Grade VIII, No G-5636) from *Escherichia coli* (EC 3.2.1.23) was obtained from Sigma. The standard enzyme solution was made by dissolving lyophilized enzyme (1.15 mg) in 0.1M sodium phosphate buffer (930 μL) and then adding 30mM dithiothreitol in D_2O (35 μL) and 30mM $Mg(NO_3)_2$ in D_2O (35 μL). The solution was stored at 3–5°. 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 methyl β-lactoside (4.00 mg, 0.0112 mmol) in 0.1M sodium phosphate buffer in D_2O (930 μL), then adding 30mM dithiothreitol in D_2O (35 μL) and 30mM $Mg(NO_3)_2$ in D_2O (35 μL). The solution was brought to 27°, 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 n.m.r. 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[20] in order to determine the rate constants V_0 and $T_{\frac{1}{2}}$.

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