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Note

Specificity of α- and β-D-galactosidase towards analogs of D-galactopyranosides modified at C-4 or C-5

JEONG E. NAM SHIN, ASAFU MARADUFU, JEAN MARION, AND ARTHUR S. PERLIN
Department of Chemistry, McGill University, Montreal, Quebec H3C 3G1 (Canada)
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In examining[1,2] the stereochemistry of D-galactose oxidase, it was found that some 4-deoxy analogs of methyl β-D-galactopyranoside (**1**), particularly the 4-amino (**2**) and 4-fluoro (**3**) derivatives[3], are relatively good substrates of the enzyme. The same compounds have now been used to assess the influence of changes in the substituent at C-4 of D-galactose on the substrate specificity of β-D-galactosidase. As a complement to this study, analogous glycosides (**6**, **7**, and **8**) in the α-D series were synthesized, and tested with an α-D-galactosidase. The effect of replacing the ring-oxygen atom by a sulfur atom was also examined, using the recently synthesized[4] methyl 5-thio-β- and -α-D-galactopyranosides (**4** and **9**, respectively).

RESULTS AND DISCUSSION

Enzyme specificity and inhibition of the D-*galactosidases.* — Tests of the susceptibility to enzymic hydrolysis were performed with the analogs of methyl α- and β-D-galactopyranoside listed in Table I. The enzymes used were a β-D-galactosidase from *Escherichia coli* and an α-D-galactosidase from *Aspergillus fumigatus*, and the reaction mixtures were monitored for release of aldose by colorimetric or chromatographic methods, or both. As shown, not a single one of the 4-deoxy analogs, or of the 5-thio analogs, proved to be a substrate for either enzyme. These negative results are in contrast to the earlier finding[2] that compounds **2** and **3** are substrates for D-galactose oxidase, as well as to the fact, observed in the present study, that this enzyme also oxidizes methyl 5-thio-α-D-galactopyranoside (**9**). Hence, the D-galactosidases are far less adaptable than the oxidase to replacement of the axial, 4-hydroxyl group or the ring-oxygen atom by other atoms or functional groups. However, they are readily able[5] to accommodate gross changes at C-5; *i.e.*, α- and β-L-arabinopyranosides (H instead of CH_2OH)[6,7] and α- and β-D-fucopyranosides (CH_3 instead of CH_2OH)[8,9] serve as substrates. Adaptation to a change at C-2 is also possible, as shown by the hydrolysis of methyl 2-deoxy-α-D-*lyxo*-hexopyranoside (**10**) by α-D-

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TABLE I

SUBSTRATE SPECIFICITIES OF α- AND β-GALACTOSIDASES

Compound	*Formula number*	*Conc. (mM)*[a]	*Enzyme*	*Time (h)*	*Hydrolysis (%)*
β-D-Galactopyranoside			*β-D-Galactosidase (units)*		
Methyl	**1**	6.8	25	0.3	82
4-amino-4-deoxy-	**2**	22.9	200	12	0
4-chloro-4-deoxy-		11.8	100	6	0
4-deoxy-4-fluoro-	**3**	26.0	100	14	0
4-thio-		4.0	150	6	0
5-thio-	**4**	18.0	100	14	0
4-Nitrophenyl	**5**	0.1	5	0.1	88
α-D-Galactopyranoside			*α-D-Galactosidase (mg)*		
Methyl	**6**	8.3	0.7	20	100
4-amino-4-deoxy-	**7**	18.8	1.6	20	0
4-deoxy-4-fluoro-	**8**	22.2	1.8	24	0
5-thio-	**9**	51.5	2.2	20	0
2-deoxy-	**10**	11.5	0.7	20	66

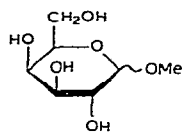
[a]Of β anomers in 0.1M phosphate buffer (pH 7.0 at 37°), and of α anomers in 0.05M citrate buffer (pH 4.5 at 50°).

galactosidase (see Table I), although 2,4-dinitrophenyl 2-chloro-2-deoxy-β-D-galactopyranoside is not affected[10] by β-D-galactosidase.

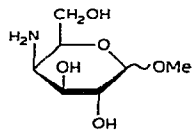
Other experiments were conducted to examine the possibility that some of the D-galactoside analogs function as inhibitors. From the rate of liberation of 4-nitrophenol from 4-nitrophenyl β-D-galactopyranoside (**5**) by β-D-galactosidase in the presence of the 4-amino (**2**) and 4-fluoro (**3**) glycosides, it was determined that the latter derivative is a competitive inhibitor (K_I = 23.5 mM), whereas the amino derivative is a non-competitive inhibitor (K_I = 5.2 mM). In these experiments, K_M was found to be 300 μM. Also, the rate of hydrolysis of **5** was unaffected by the presence of a 20-fold concentration of the methyl glycoside **1**, whereas it has been reported[11] that butyl β-D-galactopyranoside is an inhibitor of the hydrolysis of 2,4-dinitrophenyl β-D-galactoside.

As the fluorine atom in **3** is not an efficient proton-acceptor for hydrogen bonding[12], the 4-hydroxyl group of the "natural" substrate may function in this role (or as donor) in the enzyme–substrate complex*. Consequently, the 4-amino glycoside **2** may be relatively more effective because of its ability to engage in hydrogen bonding at the required location (position 4), either with the free enzyme or with the enzyme–substrate complex. Accepting that conformational changes in the β-D-galactosidase are necessary[13,14] in order to align its catalytic groups correctly, it

*This does not appear[2] to be an important role of OH-4 in reactions of D-galactose oxidase.



1 (β)
6 (α)



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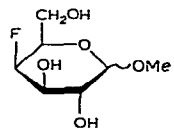
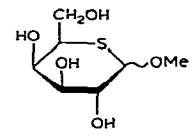
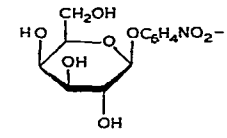


3 (β)
8 (α)



4 (β)
9 (α)



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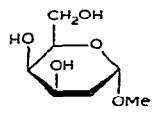

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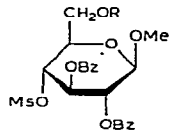
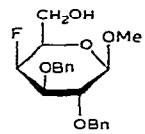


11 R = Tr
12 R = Bn
13 R = H

Tr = Ph_3C
Bn = $PhCH_2$



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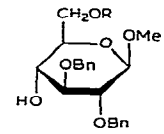
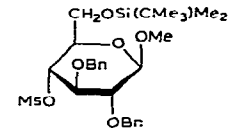


15 R = H
16 R = $Si(CMe_3)Me_2$



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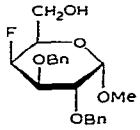

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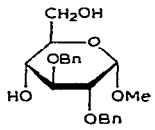

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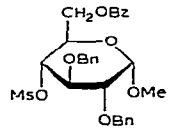

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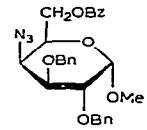

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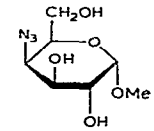

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appears that the 4-hydroxyl group of D-galactose uniquely satisfies both the spatial and hydrogen-bonding requirements of the activated enzyme.

Although the axial, 4-hydroxyl group and other general conformational features of the D-galactopyranosides are maintained[4] in the 5-thio-D-galactosides (**4** and **9**), the C–S bond is substantially longer than the C–O and, where these bonds are involved, differences in torsional angles are also expected[15]. Possibly, the D-galactosidases are unable to accommodate changes of this magnitude in the substrate. As an alternative consideration, if hydrogen bonding between the enzyme and the ring-oxygen atom of the sugar is critical, the hydrogen bond should be weakened by replacing the ring heteroatom by the (less electronegative) sulfur atom. A third possible contribution to the inertness of **4** and **9** as substrates could be related to dissociation of the enzyme–substrate complex. When, as is widely accepted[5,13], the enzyme–glycoside, covalent intermediate has been formed, dissociation *via* a glycosyl cation should take place less readily when the substrate is a thio sugar, by analogy[16] with the lower rate of solvolysis of 2,3,4-tri-*O*-acetyl-5-thio-D-xylopyranosyl bromide than of its oxygen analog.

Synthesis of 4-deoxy analogs of methyl α- and β-D-galactopyranoside. — In an earlier synthesis[3] of methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (**3**), a step involving the reaction of tetrabutylammonium fluoride with methyl 2,3-di-*O*-benzyl-4-*O*-(methylsulfonyl)-6-*O*-trityl-β-D-glucopyranoside (**11**) (or the related brosyl ester was found to take place in only 30% yield. Attempts to improve upon this yield in the present study by using phase-transfer catalysis[17] were unsuccessful; *e.g.*, the reaction of **11**, or the 6-*O*-benzyl derivative (**12**), with potassium fluoride complexed

by 18-crown-6 in *N,N*-dimethylformamide at 120° gave intractable mixtures of products. However, a marked enhancement of the fluorination step was effected by prior removal of the trityl group in **11**, giving the derivative (**13**) unsubstituted at O-6, which gave a 70% yield of methyl 2,3-di-*O*-benzyl-4-deoxy-4-fluoro-β-D-galactopyranoside (**14**) on treatment with tetrabutylammonium fluoride. This improvement accords with the well known fact[18] that SN2 displacement of secondary sulfonyloxy groups of hexopyranosides is highly sensitive to steric factors, in this instance associated with bulky substituents on O-6. Therefore, a modified route to **3** was devised in which a 6-*O*-(*tert*-butyldimethylsilyl) group (*t*-$BuMe_2Si$) was introduced, instead of the 6-*O*-trityl group of **11**; *i.e.*, methyl 2,3-di-*O*-benzyl-β-D-glucopyranoside (**15**) was selectively converted into the 6-*O*-(*t*-$BuMe_2Si$) derivative (**16**), and then **16** into the corresponding methanesulfonate (**17**). Consequently, because an *O*-(trialkylsilyl)substituent is readily displaced by fluoride ion[19], the protecting group at O-6 of **17** was removed during the subsequent introduction of the 4-fluorine atom, and a 72% yield of **14** was obtained. The same approach was used in the synthesis of methyl 4-deoxy-4-fluoro-α-D-galactopyranoside (**8**).

^{13}C-N.m.r. data obtained for **3** and **8**, as well as for their 2,3-di-*O*-benzyl precursors (**14** and **18**), are presented in Table II. The chemical shifts for **3** and **8** are close to those[20] of methyl β- and α-D-galactopyranoside, respectively, aside from that of C-4 which, as expected, is very strongly deshielded in both anomers, by ~20 p.p.m.; hence, neighboring carbon atoms appear to be virtually unaffected by the presence of the fluorine atom. It is noteworthy that the assignment of ^{13}C signals in these spectra was facilitated by the ^{13}C–^{19}F coupling observed, because the magnitude of these couplings decreases so distinctively with the number of intervening bonds.

For the synthesis of methyl 4-amino-4-deoxy-α-D-galactopyranoside (**7**), methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside (**19**) was selectively benzoylated at O-6, and the 6-benzoate treated with methanesulfonyl chloride, affording **20**. Upon

TABLE II

^{13}C-N.M.R. DATA[a] FOR 4-DEOXY-4-FLUORO DERIVATIVES OF D-GALACTOSE

	3[b]	**8**[b]	**14**[b]	**18**[c]
C-1	104.9	100.9	103.0	97.1
C-2	72.0	69.6 (2.6)	77.4	74.9
C-3	73.0 (17.9)	69.7 (18.0)	71.9 (17.6)	68.2 (17.6)
C-4	90.9 (178.2)	91.8 (177.4)	84.4 (182.6)	85.8 (181.6)
C-5	75.0 (17.6)	71.9 (17.6)	77.4 (17.9)	74.5 (17.6)
C-6	61.3 (5.1)	61.5 (5.5)	59.1 (5.8)	59.2 (5.9)
OMe	58.6	56.7	55.6	53.4
OCH_2	—	—	77.4, 73.6	71.7, 70.6
Ph	—	—	126.1–137.0	125.9–137.0

[a]^{13}C-Chemical shifts (δ) and, in parentheses, observed coupling (Hz) between ^{13}C and ^{19}F. [b]Solvent, D_2O. [c]Solvent, C_6D_6.

displacement with azide ion, the latter was converted into the 4-azido-α-D-*galacto* derivative **21**, and this afforded the known[21] methyl 4-azido- (**22**) and 4-amino-α-D-galactopyranoside (**7**).

EXPERIMENTAL

General methods. — Solutions were usually evaporated below 40° under diminished pressure. Optical rotations were determined at room temperature, for solutions in a 1-dm tube, with a Carl Zeiss polarimeter (Model 367732). I.r. spectra were recorded for films on AgCl or NaCl discs, or for KBr pellets, with a Unicam SP-200G grating spectrophotometer. Electronic spectra were recorded with a Unicam SP-800 spectrometer. Microanalyses were performed by C. Daessle, Montreal. Plates of Silica Gel-G were used for t.l.c., and the developing solvent was 3:2:1 1-propanol–acetic acid–water. Silica Gel for column chromatography (0.08 mm particle size) was obtained from Macherey–Nagel and Co. Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard F and M 402 gas chromatograph, using an OV-225 column. Proton magnetic resonance spectra were recorded with a Bruker WH-90 spectrometer, and ^{13}C-n.m.r. spectra were recorded at 22.6 MHz with the same spectrometer. Chemical shifts (δ) are reported with reference to tetramethylsilane.

Enzymic reactions. — β-D-Galactosidase (EC 3.2.1.23) of *Escherichia coli* was purchased from Worthington Biochemical Corporation, and also from Aldrich Chemical Co., Inc. The α-D-galactosidase from *Aspergillus fumigatus* was a gift from E. T. Reese. The release of free aldose by enzymic hydrolysis was assayed colorimetrically (at 500 nm) with a copper–molybdate reagent[22], or by g.l.c. of the derived alditol peracetate. Enzymic digests were also examined by t.l.c. and, in some instances, by ^{1}H-n.m.r. spectroscopy. Reactions of the β-D-galactosidases were conducted at 37° in 0.1M phosphate buffer, pH 7.0, and those of the α-D-galactosidase, at 50° in 0.05M citrate buffer, pH 4.5, as recommended by E. T. Reese (personal communication).

Inhibition tests. — A solution of β-D-galactosidase in 0.1M phosphate buffer (pH 7.0; 1.0 mL) was incubated at 37° with a solution (9.0 mL) of the appropriate inhibitor (4.4 mg of **2**, or 7.1 mg of **3**) and 4-nitrophenyl β-D-galactopyranoside (118–295 μg). Samples withdrawn periodically were made alkaline with 0.1M potassium carbonate, and assayed for 4-nitrophenol by measurement of the optical absorbance at 420 nm. Plots of optical absorbance *versus* time were nominally linear over the interval 2–9 min. Values of K_M and K_I were estimated from Lineweaver–Burk plots.

*Methyl 2,3-di-*O*-benzyl-6-*O*-(*tert*-butyldimethylsilyl)-4-*O*-(methylsulfonyl)-β-D-glucopyranoside* (**17**). — A solution of methyl 2,3-di-*O*-benzyl-β-D-glucopyranoside[23] (**14**) (1.91 g) and *tert*-butylchlorodimethylsilane (0.86 g, 1.1 equiv.) in dry pyridine (15 mL) was stirred for 14 h at room temperature. Methanesulfonyl chloride (1 mL) was added, stirring was continued for an additional 14 h, and then ice–water was

added. The mixture was extracted with chloroform, and the extract was washed successively with water, 2M hydrochloric acid, water, saturated sodium hydrogencarbonate, and water, dried (anhydrous sodium sulfate), and evaporated, to give a solid (2.8 g, 97%). After recrystallization from ethanol, the product had m.p. 74–75°, $[\alpha]_D$ +27.2° (*c* 3.6, chloroform).

Anal. Calc. for $C_{28}H_{42}O_8SSi$: C, 59.3; H, 7.5; S, 5.7. Found: C. 59.3; H, 7.4; S, 5.6.

Methyl 2,3-di-O-benzyl-4-deoxy-4-fluoro-β-D-galactopyranoside (**14**). — A solution of **17** (3.0 g) and tetrabutylammonium fluoride (5.2 g, 3 equiv.) in dry *N,N*-dimethylformamide (10 mL) was heated for 48 h at 120°. The solvent was distilled off (100°/22 μm Hg), the residue was extracted with chloroform, and the extract was washed with water, dried (anhydrous sodium sulfate), and evaporated, to give crystalline **14** (1.37 g); m.p., after recrystallization from chloroform–petroleum ether, 100–102°, $[\alpha]_D$ −11.3° (*c* 3.2, chloroform); lit.[3] m.p. 105–106°, $[\alpha]_D$ −15.6° (*c* 2, chloroform); for the ^{13}C-n.m.r. data, see Table II.

Methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (**3**). — To a solution of **14** (1.85 g) in ethanol (15 mL) was added palladium-on-charcoal catalyst (0.61 g), and the suspension was stirred for 14 h under an atmosphere of hydrogen. The solids were filtered off, and the filtrate was evaporated, to give crystalline **3** (0.93 g, 100%); m.p., after recrystallization from ethanol–ethyl acetate, 153°, $[\alpha]_D$ −20.7° (*c* 1.3, water); lit.[3] m.p. 155–156°, $[\alpha]_D$ −21.3° (*c* 1, water); for the ^{13}C-n.m.r. data, see Table II.

Methyl 2,3-di-O-benzyl-O-(tert-butyldimethylsilyl)-4-O-(methylsulfonyl)-6-α-D-glucopyranoside. — This compound was synthesized from methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[24] by the sequence of reactions described for the synthesis of **17**. The product was a chromatographically pure syrup; $[\alpha]_D$ +58.8° (*c* 1.9, chloroform).

Methyl 2,3-di-O-benzyl-4-deoxy-4-fluoro-α-D-galactopyranoside (**18**). — Prepared from the preceding compound by the procedure described for the conversion of **17** into **14**, syrupy **18** was chromatographically pure; $[\alpha]_D$ +41.0° (*c* 2.6, chloroform); for the ^{13}C-n.m.r. data, see Table II.

Methyl 4-deoxy-4-fluoro-α-D-galactopyranoside (**8**). — On removal of the *O*-benzyl groups of **18** by catalytic hydrogenolysis as described for **14**, crystalline glycoside **8** was obtained; m.p., after recrystallization from methanol–ethyl acetate, 102–103°, $[\alpha]_D$ +144.5° (*c* 1.7, methanol); lit.[25] m.p. 99–101°, $[\alpha]_D$ +148°.

Methyl 6-O-benzoyl-2,3-di-O-benzyl-4-O-(methylsulfonyl)-α-D-glucopyranoside (**20**). — A solution of benzoyl chloride (3 mL) in chloroform (20 mL) was added dropwise to a stirred solution, at −15°, of methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside (**19**) (7 g) in pyridine (70 mL). After 8 h, the mixture was kept for 18 h at room temperature, methanesulfonyl chloride (1.5 mL) was added, and, 18 h later, ice–water was added, followed by chloroform. The chloroform extract was washed successively with water, 2M hydrochloric acid, water, saturated sodium hydrogencarbonate, and water, dried (anhydrous sodium sulfate), and evaporated, to give a

solid residue. After purification by column chromatography on Silica Gel with 50:1 benzene–ether as the eluant (yield, 4.0 g), the product was recrystallized from toluene–petroleum ether; m.p. 116–117°, $[\alpha]_D$ +47° (*c* 1.5, chloroform).

Anal. Calc. for $C_{29}H_{32}O_9S$: C, 62.6; H, 5.8. Found: C, 62.8; H, 6.0.

*Methyl 4-azido-6-*O*-benzoyl-2,3-di-*O*-benzyl-α-*D*-galactopyranoside* (**21**). — A solution of **20** (6.7 g) in *N,N*-dimethylformamide (60 mL), containing sodium azide (4 g) in suspension, was boiled under reflux for 100 h. Water and chloroform were introduced, and the chloroform layer was separated, washed with water, dried (anhydrous sodium sulfate) and evaporated, to give a solid product; m.p., after recrystallization from ethanol, 97–98°, $[\alpha]_D$ −8.5° (*c* 1.4, chloroform); ν_{max} 2150 cm^{-1} (N_3).

Anal. Calc. for $C_{28}H_{29}N_3O_6$: C, 66.8; H, 5.8. Found: C, 66.6; H, 5.7.

*Methyl 4-azido-4-deoxy-α-*D*-galactopyranoside* (**22**). — On *O*-debenzoylation with sodium methoxide and *O*-debenzylation by hydrogenolysis in the presence of palladium, compound **21** afforded crystalline **22**; m.p., after recrystallization from 2-propanol–benzene, 154–155°, $[\alpha]_D$ +115° (*c* 1.9, methanol) {lit.[21], m.p. 153–155°, $[\alpha]_D$ +120° (*c* 0.5, methanol)}, ν_{max} 2150 cm^{-1} (N_3).

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