The preferred rotamer about the C_5—C_6 bond of D-galactopyranoses[1] and the stereochemistry of dehydrogenation by D-galactose oxidase

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^{1}H nuclear magnetic resonance studies on D-galactopyranoses with chirally deuterated hydroxymethyl groups revealed that the preferred rotamer about the C_5—C_6 bond of the D-galactopyranoses was not *tg* but *gt*. Studies on the stereochemistry of dehydrogenation of D-galactose oxidase from *Dactylium dendroides* with chirally pure deuterated methyl β-D-galactopyranosides showed that there were two mechanisms for the enzymatic reaction: an efficient *pro-S* hydrogen atom specific oxidation and a far less efficient non-specific or *pro-R* specific oxidation.

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Des études de résonance magnétique nucléaire du ^{1}H sur des D-galactopyrannoses portant des groupements hydroxyméthyles deutérés d'une façon chirale révèlent que le rotamère privilégié autour de la liaison C_5—C_6 des D-galactopyrannoses n'est pas *tg* mais plutôt *gt*. Des études sur la stéréochimie de la déshydrogénation, par l'oxydase du D-galactose provenant du *Dactylium dendroides*, de β-D-galactopyrannosides de méthyles deutérés et purs d'un point de vue chiral ont démontré qu'il existe deux mécanismes pour la réaction enzymatique; une oxydation spécifique de l'atome d'hydrogène *pro-S* qui est efficace et un oxydation non-spécifique ou spécifique *pro-R* qui est beaucoup moins efficace.

[Traduit par la revue]

Introduction

A complete understanding of the interaction between carbohydrates and proteins (enzymes, antibiotics, and lectins) is to a large extent dependent on the information available about the preferred conformation of the carbohydrate molecule in solution (1, 2). For example, the current stereochemical concept that D-galactose residues favor the *tg* rotamer as shown in Fig. 1 (3) in the binding sites of D-galactosyl binding proteins (1) has been based mainly on the conformational analyses of methyl β-D-galactopyranoside by Lemieux and co-workers (1, 4) and of acetylated D-galactoses by Hall *et al.* (5) and Perlin and co-workers (6). The assignment of the preferred rotamer about the C_5—C_6 bond of D-galactose and its derivatives has been a controversial problem. For example, Yamana (7), by a chiroptical method, reported that the preferred rotamer was *gg*. Hall, by an ^{1}H nmr study on D-galactopyranose peracetate (5), reported it to be *tg*. On the other hand, De Bruyn suggested it to be *tg* (8) and Lemieux first assigned it to be *gt*, based on both ^{1}H nmr and chiroptical studies (9), and later corrected it to be *tg*, based on a detailed ^{1}H nmr study on methyl β-D-galactopyranoside (4). Recently Lew and Nakanishi assigned the preferred rotamer of methyl D-galactopyranoside perbenzoates to be *tg* on the basis of their "dibenzoate chirality rule" (10). Therefore *tg* has been accepted as the preferred rotamer of both acylated and OH-free D-galactopyranoses. The difficulty in the conformational analysis of the C_5—C_6 bond of D-galactopyranose by ^{1}H nmr has been ascribed to the difficulty of differentiating unequivocally the two protons, H6*proR* and H6*proS*, and of obtaining the precise coupling constants, $J_{H5,H6proR}$ and $J_{H5,H6proS}$, which is the key information needed for the study. Our recent development of syntheses of sugars with chirally deuterated hydroxymethyl groups has enabled us to differentiate the two protons and obtain the coupling constants (11–14).[4] In this paper, we would like to describe the results of our conformational analyses and the stereochemistry of oxidation by D-galactose oxidase from *Dactylium dendroides* using the chirally deuterated D-galactopyranoses as substrates.

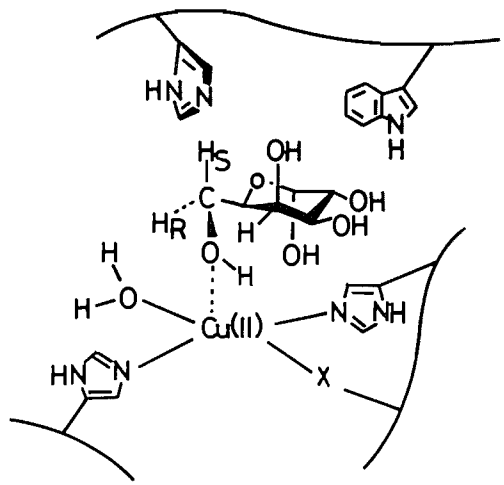

FIG. 1. The current concept of the active site of D-galactose oxidase.

Results and discussion

Previously we reported the synthesis of (6*R*)- and (6*S*)-(6-$^2H_1$)-D-galactoses through photobromination (15) of 1,6-anhydro-2,3,4-tri-*O*-benzoyl-D-galactopyranose **1** followed by

[1]Part of this study has been already reported in ref. 13.
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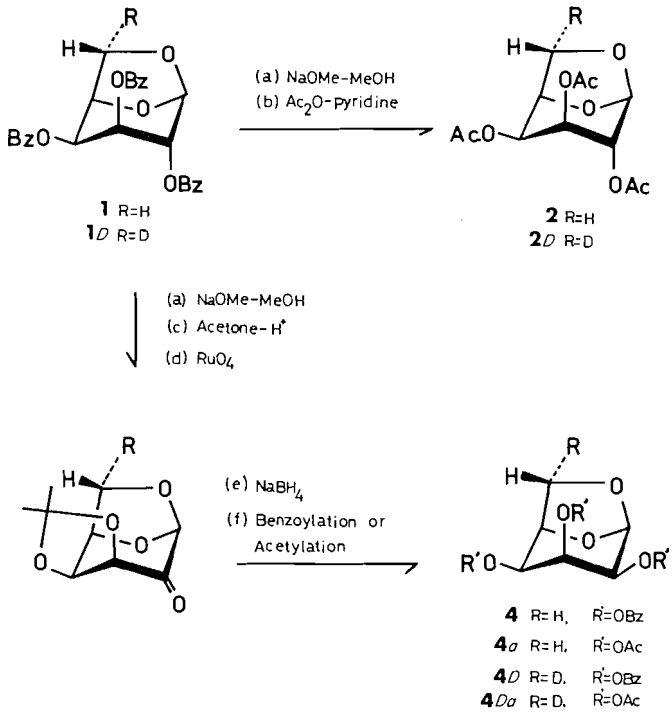
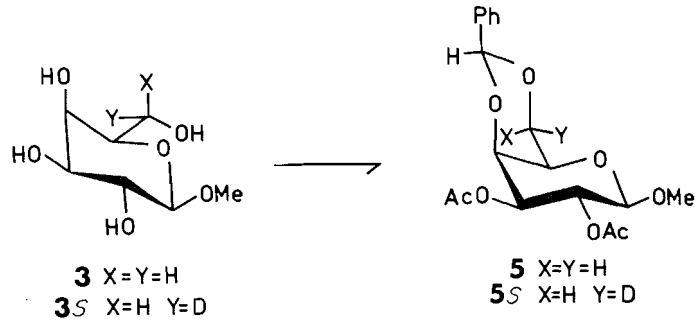
[4]The authors are very grateful to a referee for pointing out the errors in assigning the ^{1}H nmr spectrum of 1,6-anhydro-D-glucopyranose (11). The correction will be reported in another manuscript (see footnote 5).



SCHEME 1



SCHEME 2

tri-*n*-butyltindeuteride reduction of the resulting *exo*-bromide (12). Our assignments of absolute configuration of the specifically deuterated galactose derivatives have been based on the "assumption" that protons at C-6 of 1,6-anhydrohexopyranose 2,3,4-tri-*O*-benzoates, which resonate at higher field and have larger coupling constants with H-5 than the other proton at C-6, could be assigned to H-6*exo* (12), because the same findings have been reported for 1,6-anhydro-D-hexopyranoses and for their 2,3,4-tri-*O*-acetate (16, 17).

In the present paper, several other pieces of evidence that confirm the correctness of our previous assignments are presented.

Compound **1***D*, which we had assigned to (6*S*) (12), was converted to the acetate **2***D* by a conventional method (sodium methoxide in MeOH and then acetylation) (Scheme 1). In the ^{1}H nmr spectra of **2***D* the signal at 3.72 ppm, which had been assigned to H-6*exo* of nondeuterated **2** by Černý (17), disappeared completely and the doublet at 4.347 ppm, which had been assigned to H-6*endo* (17), appeared at 4.326 ppm (0.021-ppm upfield shift by deuterium isotope effect (18, 19)) as a singlet. The result supports the correctness of our previous assignments.

Next, our methyl (6*R*)-(6-^{2}H$_1$)-β-D-galactopyranoside **3***R* and methyl (6*S*)-(6-^{2}H$_1$)-β-D-galactopyranoside **3***S* were converted to the corresponding acetates **3***Ra* and **3***Sa*, respectively. The partial ^{1}H nmr spectra of **3***Ra* and **3***Sa* and nondeuterated **3***a* are shown in Fig. 2. The assignments of H6*proR* and H6*proS* are consistent with those reported by Perlin and co-workers (6). This result also supports the validity of our previous assignments. The ^{1}H nmr spectra of both **3***Ra* and **3***Sa* show the presence of a very small amount of **3***a*. This amount depends on the quality of tin deuteride used. As is observed clearly in Fig. 2, deuteration causes a 0.015-ppm upfield shift of H6*proR*, a 0.017-ppm upfield shift of H*proS*, and a 0.004-ppm upfield shift of H-5; however, *deuteration has little effect on the coupling constants*. Similar deuterium isotope-induced chemical shifts of protons have been reported by Hall and Wong (18).

Another method of assignment of H-6*exo* in 1,6-anhydrohexopyranoses is measurement of the "W" arranged long-range coupling between H-6*exo* and H-4, since only H-6*exo* can enjoy the "W" type long-range coupling (17, 20). Irradiation at 5.74 ppm of **1** sharpened the quartet of broad signals at 3.85 ppm, which we assigned to H-6*exo* (12), indicating the presence of long-range coupling in the signal of H-6*exo*. However, the result does not give us an unequivocal assignment of H-6*exo* because the chemical shifts of H-1 and H-4 are almost the same at 5.74 ppm, and long-range coupling between H-1 and H-6*exo* has been reported (16).

Therefore, compound **1** was transformed to 1,6-anhydrotalopyranose-2,3,4-tri-*O*-benzoate **4** (mp 178°C, $[\alpha]_D^{21}$ −58° (*c* 0.1, $CHCl_3$)) and tri-*O*-acetate **4***a* by the established method (20) (Scheme 1). The "W" arranged long-range coupling between H-6*exo* and H-4 of **4***a* has already been reported by Horton and Jewell (20) and a "W" arranged long-range coupling is observed between H-6 (quartet of a small doublet at 3.993 ppm) and H-4 (triplet of small doublets at 5.608 ppm) of **4**. Therefore the H-6 signal at 3.993 ppm, which has a larger coupling with H-5 than the other H-6 signal at 4.78 ppm, is assigned to H-6*exo*. Compound **1***D* was also converted to **4***D* and **4***Da*. The ^{1}H nmr spectra of **4***D* and **4***Da* showed complete disappearance of the signals for H-6*exo* protons. Therefore **4***D* and **4***Da* are unequivocally assigned to be the (6*S*)-(6-^{2}H$_1$) derivatives of **4** and **4***a*, respectively. Since all reactions used for transformations proceed with retention of configuration at C-6, **1***D* is definitively assigned to be the (6*S*)-(6-^{2}H$_1$) derivative of **1**.

To confirm further the assignment of absolute configuration of these C-6 chirally deuterated galactoses, **3** and **3***S* were converted into methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosides **5** and **5***S*, respectively, with the *gg* fixed conformation (Scheme 2).

In the ^{1}H nmr spectrum of **5**, it is reasonable to assign the more deshielded H-6 proton to H6*proS* and the more shielded one to H6*proR* because H6*proS* and H6*proR* are in equatorial and axial dispositions, respectively, with respect to the 4,6-*O*-benzylidene ring structure. Thus, in the ^{1}H nmr spectrum of **5**, the H-6 proton signals at 4.34 ppm (dd, 1.7 and 12.4 Hz) and 4.07 ppm (dd, 1.7 and 12.4 Hz) are assigned to H6*proS* and H6*proR*, respectively. In the spectrum of **5***S*, the lower field signal in that of **5** disappeared and the other appeared as a doublet at 4.06 ppm (1.7 Hz). These results also confirm our previous assignments.

All these pieces of evidence strongly confirm the assignments

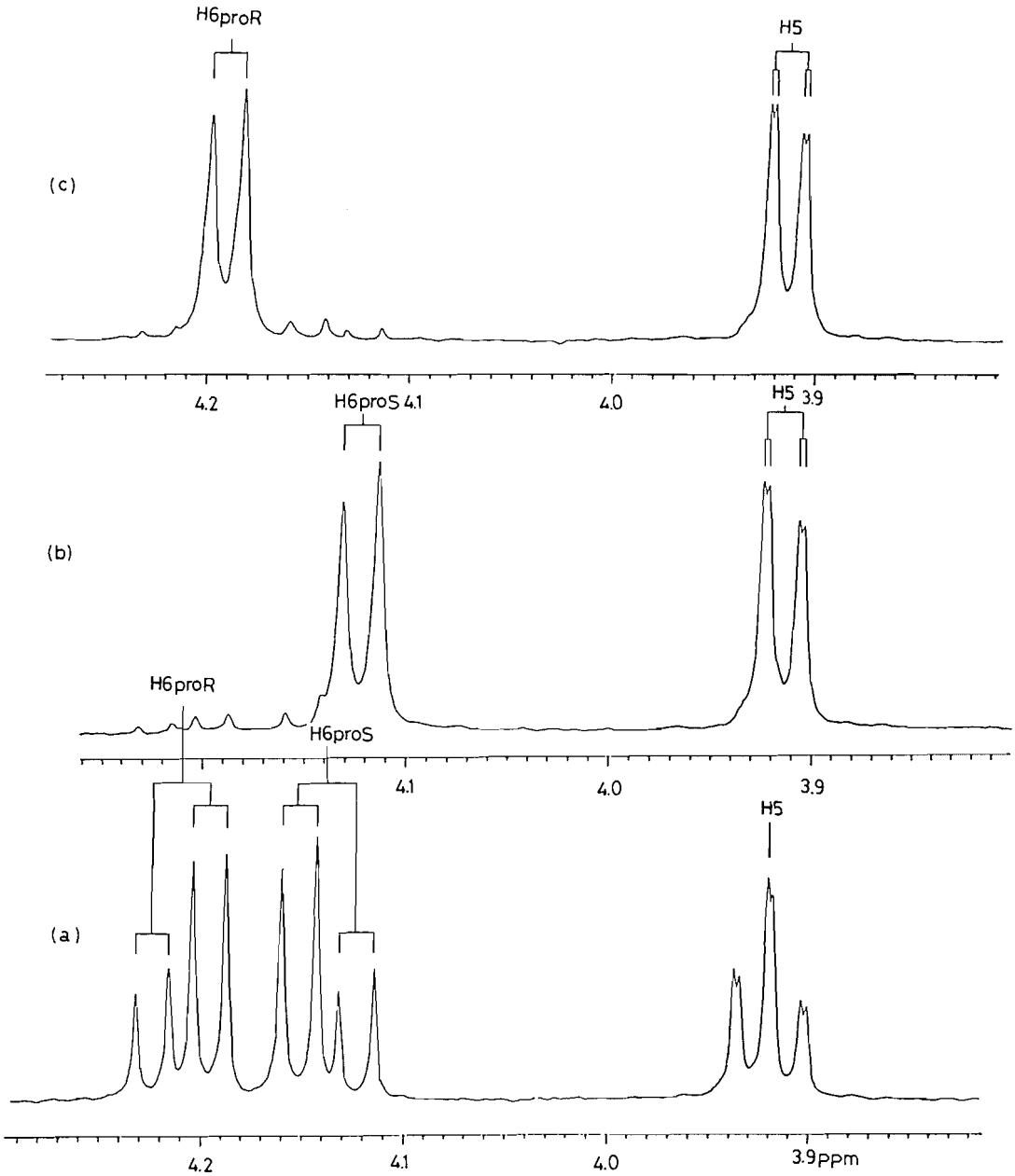


FIG. 2. The ^{1}H nmr spectra of (*a*) methyl tetra-*O*-acetyl-β-D-galactopyranoside **3***a*, (*b*) methyl (6*R*)-(6-$^2H_1$)-tetra-*O*-acetyl-β-D-galactopyranoside **3***Ra*, and (*c*) methyl (6*S*)-(6-$^2H_1$)-tetra-*O*-acetyl-β-D-galactopyranoside **3***Sa* at 400 MHz in $CDCl_3$.

of absolute configuration of the two specifically 6-deuterated galactoses made by us.[5]

The ^{1}H nmr spectra of methyl β-D-galactopyranoside **3**, methyl (6*R*)-(6-$^2H_1$)-β-D-galactopyranoside **3***R*, and methyl (6*S*)-(6-$^2H_1$)-β-D-galactopyranoside **3***S* are shown in Fig. 3 to demonstrate how the chirally deuterated sugars are useful for the study. The complex signals of H-5, H-6*proR*, H-6*proS* of **3** are dramatically simplified in the spectra of deuterated **3***R* and **3***S*. Therefore it is very easy to assign the signals and to measure $J_{H5,H6proR}$ and $J_{H5,H6proS}$. Deuterium-isotope induced upfield chemical shifts (18, 19) are also observed in these spectra. Therefore the chemical shifts of H6*proR* and H6*proS* of nondeuterated **3** should be about 0.015–0.02 ppm lower than those obtained with deuterated derivatives **3***R* and **3***S*.

So far as the data obtained by us is concerned, *a deuterium isotope effect on the coupling constants has not been observed*; therefore the coupling constants obtained with deuterated derivatives can be considered those of normal compounds.

The ^{1}H nmr parameters of **3**, **3***R*, **3***S* and their acetates **3***a*, **3***Ra*, **3***Sa*, together with those of **3** reported by Bock and Thøgersen (21) and De Bruyn (8), are listed in Table 1. Differences between our chemical shifts and theirs arise from differences in the conditions of measurement, for example the different internal standards. We used 3-(trimethylsilyl)propane-

[5]We are now very confident that the assignments of H-6 protons of 1,6-anhydrohexopyranoses can be made by the rule that the *exo* protons resonate at higher field and have larger coupling with H-5 than do the *endo* protons. This is discussed in a paper that has been submitted to the Journal of Carbohydrate Chemistry.

TABLE 1. Comparison of the ^1H nmr parameters of methyl β-D-galactopyranoside (**3**), its (6*R*)-(6-$^2H_1$) derivative (**3***R*), and (6*S*)-(6-$^2H_1$) derivative (**3***S*) with the reported data

Chemical shifts[a] (Coupling constant)	H1 ($J_{1,2}$)	H2 ($J_{2,3}$)	H3 ($J_{3,4}$)	H4 ($J_{4,5}$)	H5 ($J_{5,6R}$)	H6*proR* ($J_{6R,6S}$)	H6*proS* ($J_{5,6S}$)	OMe
De Bruyn and Anteunis (8)					3.70 (3.5)	3.76	3.83 (8.5)	
Bock and Thøgersen (21)	4.20 (8.0)	3.39 (10.0)	3.53 (3.8)	3.81 (0.8)	3.57 (7.6)	3.69 (11.2)	3.64 (4.4)	3.45
3[b]	4.3245 (8.0)	3.5081 (10.0)	3.6532 (3.5)	3.9291 (0.9)	[3.705 [(8.2)	3.802 (11.8)	3.763][d] (3.6)][d]	3.5820
3*S*[b]	4.3245 (8.0)	3.5082 (10.0)	3.6539 (3.5)	3.9292 (0.9)	3.702 (8.0)	3.784 —	— —	3.5826
3*R*[b]	4.3242 (8.0)	3.5079 (10.0)	3.6539 (3.5)	3.9278 (0.9)	3.702 —	— —	3.744 (4.4)	3.5820
Δ **3***S* − **3**	0	+0.0001	+0.0007	+0.0001	−0.003	−0.018	—	+0.0006
Δ **3***R* − **3**	−0.003	−0.0002	+0.0007	−0.0013	−0.003	—	−0.019	0
3*a*[c]	4.4061 (7.9)	5.2072 (10.5)	5.0210 (3.4)	5.3977 (1.0)	3.9189 (6.6)	4.207	4.140 (6.8)	3.5259
3*Sa*[c]	4.4046 (7.9)	5.2070 (10.5)	5.0202 (3.4)	5.3956 (1.0)	3.9149 (6.6)	4.192 —	— —	3.5259
3*Ra*[c]	4.4053 (7.9)	5.2076 (10.5)	5.0208 (3.4)	5.3969 (1.0)	3.9149 —	— —	4.123 (7.0)	3.5267
Δ **3***Sa* − **3***a*	−0.0015	−0.0002	−0.0008	−0.0021	−0.0040	−0.015	—	0
Δ **3***Ra* − **3***a*	−0.0008	+0.0004	−0.0002	−0.0008	−0.0040	—	−0.017	+0.0008

[a](ppm) ±0.0005, Hz ±0.2.
[b]Measured at 400 MHz in D_2O at 296 K relative to internal 3-(trimethylsilyl)propanesulfonic acid sodium salt (0.0000 ppm).
[c]Measured in $CDCl_3$ relative to internal TMS (0.0000 ppm).
[d]Values obtained through ABX analyses.

TABLE 2. The chemical shifts, coupling constants, and calculated populations of rotamers of stereospecifically deuterated D-galactose derivatives and of those reported in the literature (6, 21)

						Rotamers (%)[b]								
						Equation A			Equation B			Equation C		
	Solvents	H6*R* (ppm)	H6*S* (ppm)	$J_{5,6R}$ (Hz)[a]	$J_{5,6S}$ (Hz)[a]	*gg*	*gt*	*tg*	*gg*	*gt*	*tg*	*gg*	*gt*	*tg*
α-D-Galactopyranose	D_2O	3.71	3.71	7.9	4.6	21	54	25	17	63	20	20	60	20
(21)		3.72 – 3.62		6.4	6.4	25	30	45	18	38	44	21	36	43
β-D-Galactopyranose	D_2O	3.76	3.73	7.8	4.6	22	53	25	18	62	20	21	59	20
(21)		3.70 – 3.62		7.8	3.8	27	56	17	24	66	10	27	62	11
Methyl α-D-galactopyranoside	D_2O	3.76	3.75	8.3	4.0	21	61	18	13	70	17	20	67	13
(21)		3.67	3.61	8.2	4.6	19	57	24	14	66	20	17	62	21
Methyl β-D-galactopyranoside	D_2O	3.79	3.75	8.0	4.4	22	55	23	17	65	18	21	62	18
(21)		3.69	3.64	7.6	4.4	25	52	23	21	61	18	25	57	18
Methyl tetra-*O*-acetyl-β-D-galactopyranoside	$CDCl_3$	4.19	4.12	6.6	6.8	21	30	49	13	38	59	16	37	47
(6)				6.3	7.2	21	25	54	13	32	55	16	32	52
Penta-*O*-acetyl-α-D-galactopyranose	$CDCl_3$	4.07	4.12	6.6	7.1	19	29	52	11	36	53	14	36	50
Methyl tetra-*O*-benzoyl-α-D-galactopyranoside	$CDCl_3$	4.61	4.41	—[c]	8.8	—	— tg ≅ 80 —		—			—	—	
Methyl tetra-*O*-benzoyl-β-D-galactopyranoside	$CDCl_3$	4.69	4.45	6.3	6.6	24	29	47	18	36	46	21	35	44

[a]First-order analysis, $J_{5,6}$ ± 0.2 Hz.
[b]Errors by ±0.2 Hz are *gg* ± 3, *gt* ± 3, and *tg* ± 3.
[c]The values could not be obtained because of overlapping of signals.

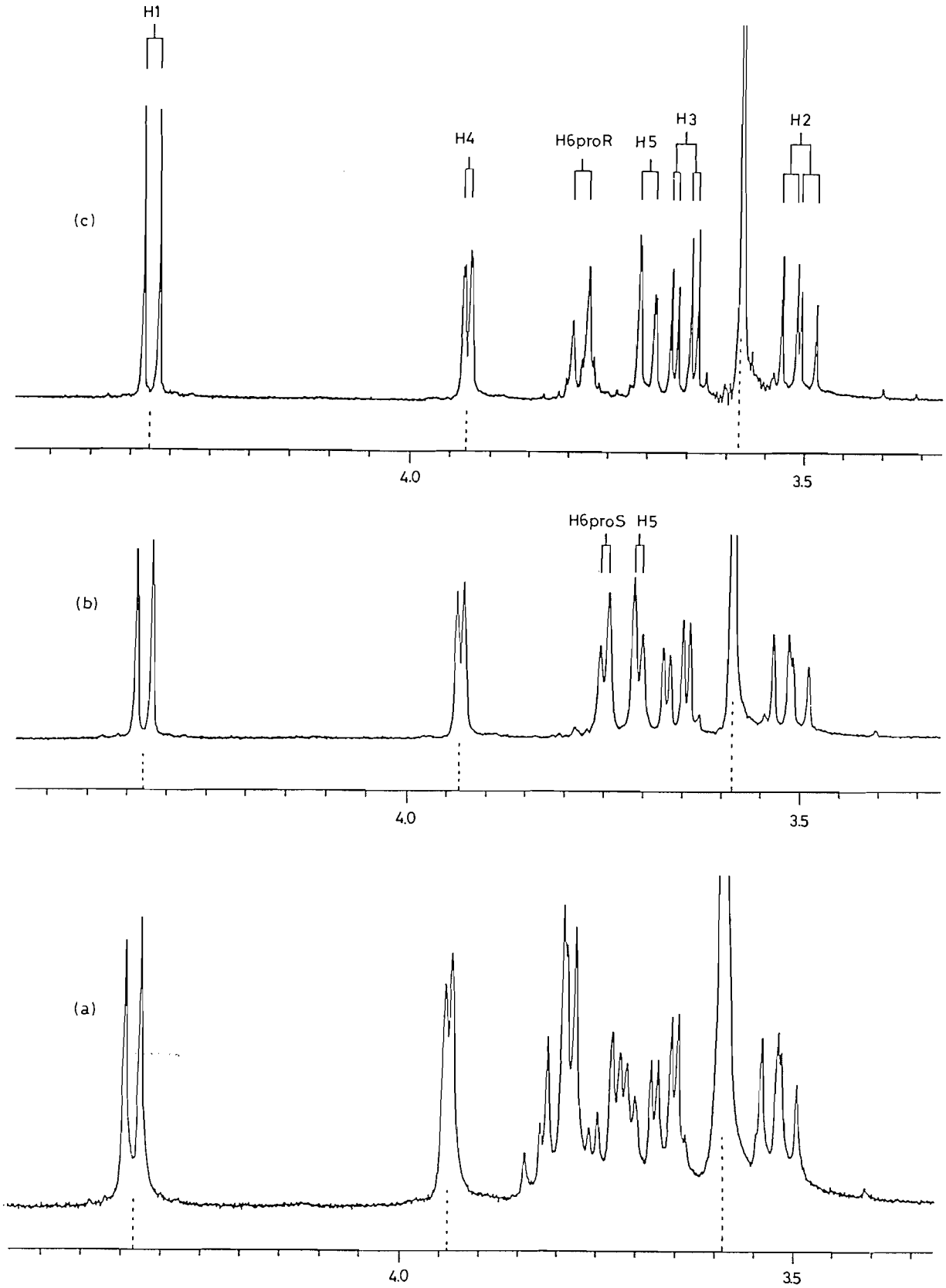


FIG. 3. The ^{1}H nmr spectra of (*a*) methyl β-D-galactopyranoside **3**, (*b*) methyl (6*R*)-(6-$^2H_1$)-β-D-galactopyranoside **3***R*, and (*c*) methyl (6*S*)-(6-$^2H_1$)-β-D-galactopyranoside **3***S* at 400 MHz in D_2O.

sulfonic acid sodium salt as 0.00 ppm and Bock and Thøgersen used acetone as 2.12 ppm.

The chemical shifts and the coupling constants by first-order analysis[6] of the stereospecifically deuterated D-galactose derivatives and those reported in the literature (6, 21), together with rough proportions of the populations of rotamers about the C_5—C_6 bond calculated by equations A devised by Rabczenko and co-workers (22), and B and C by Altona and co-workers (23, 24) are listed in Table 2. Although the two H-6 protons in the literature (21) have not been specifically assigned to H6*proR* and H6*proS*, they can be assigned unequivocally to H6*proR* and H6*proS*, respectively, by comparing their chemical shifts and coupling constants with those of the stereospecifically deuterated derivatives. Here, our value of $J_{H5,H6proS}$ of methyl β-D-galactopyranoside is revised from 6.0 (13) to 4.4 Hz, because the former value was obtained by comparing the first-order analysis of the two H-5 signals of nondeuterated compound **3** and of (6*S*)-(6-$^2H_1$) derivative **3***S* and the latter value was obtained directly from the (6*R*)-(6-$^2H_1$) derivative **3***R*.

The finding that $J_{H5,H6proS} > J_{H5,H6proR}$ in acylated derivatives and $J_{H5,HproR} > J_{H5,H6proS}$ in OH-free derivatives indicates (4, 8) that the preferred rotamer of acylated galactoses is *tg*, which is consistent with the reported results (5, 6, 10); however, the preferred rotamer of OH-free galactoses is not the previously predicted *tg* (1, 4, 6, 8), but *gt*. The same results were obtained by all three calculations except for the reported data of α-D-galactopyranose (4, 21). Although the populations of "unfavored" *gg* rotamers might be overestimated in our calculations, the not negligible contributions of *gg* will be partially rationalized in terms of the "*gauche* effect" (25).

As has been assumed (1, 2), if the conformation of the substrates in the binding sites of protein is the preferred conformation in solution, our results suggest that the *gt* rotamer should be the most plausible candidate for the galactosyl-binding protein.

The discrepancy between our result and the result previously suggested (4, 8), concerning the preferred rotamer of OH-free galactoses and chirally deuterated galactoses of high purity (99% deuterium content and no trace of diastereomer), prompted us to investigate the stereochemistry of the D-galactose oxidase reaction.

D-Galactose oxidase catalyzes the reaction:

[1] D-Galactose + O_2 → DL-galactohexodialdose + H_2O_2

The stereochemistry of D-galactose oxidase from *Polyporus circinatus* has been shown by Perlin and co-workers (6) to be a specific *pro-S* 6-hydrogen atom removal.

Methyl β-D-galactopyranoside **3**, methyl (6*R*)-(6-$^2H_1$)-β-D-galactopyranoside **3***R* (99% pure, 1% of **3**), methyl (6*S*)-(6-$^2H_1$)-β-D-galactopyranoside **3***S* (99% pure, containing 1% of **3**), and methyl (6,6-$^2H_2$)-β-D-galactopyranoside **3***DD* (99% pure, containing 1% of **3**) were used as the substrates of D-galactose oxidase (Fig. 4).

The galactose oxidase reactions were carried out under conditions similar to those reported by Perlin and co-workers (6). Acetylation and chromatographic purification (6) of the 6-aldehyde product of **3** gave three isolable products. One of

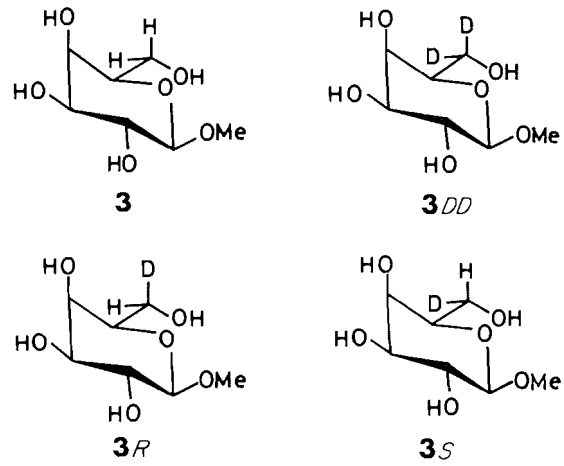


FIG. 4. Structures of substrates.

these was characterized as the known methyl 2,3-di-*O*-acetyl-L-*threo*-4-deoxyhex-4-eno-dialdopyranoside **6** (6, 26), and the others were acetylated dimers **7***a* and **7***b* of the 6-aldehyde (Fig. 5) on the basis of their ^1H nmr spectra (Table 3) and elemental analyses. The main dimer **7***a* was identified as the hexaacetate of dimeric methyl β-D-galactohexo-dialdo-1,5-pyranoside reported by Maradufu and Perlin (26).

The elemental analysis of the minor product **7***b* gave the same compositions of C, H, O as that of **7***a*. The ^1H nmr spectrum of **7***b* indicated that **7***b* had six acetyl groups and five signals that were almost the same as those of the ring protons (H_1', H_2', H_3', H_4', H_5') of the methyl D-galactopyranoside residue (ring B) of **7***a*. In **7***b*, H-4 resonates at 4.21 ppm, which is 0.19 ppm more shielded than H-4 of **7***a*; H-6′ resonates at 4.80 ppm, which is 0.21 ppm more shielded than H-6′ of **7***a* and, further, H-6 resonates at 5.91 ppm, which is 0.39 ppm more shielded than H-6 (6.22 ppm) of **7***a*. The chemical shifts of other protons of **7***b* showed little difference from those of **7***a* (in the order of −0.02 to 0.07 ppm).

All these results suggested that **7***b* has the same fundamental structure as that of **7***a*. The upfield shifts of H-4, H-6′, and H-6 indicate the change of configuration of the 6-OAc group from axial (6*R*) to equatorial (6*S*), since the change loses the 1,3-diaxial relationships between H-4, H-6′, and 6-OAc and converts H-6 from equatorial to axial configuration.

The chemical shift of H-5 of **7***b* is 3.66 ppm, which is 0.29 ppm more deshielded than that of **7***a*. Similar differences in the chemical shifts of the H-2 protons between α- and β-D-mannopyranose acetates are usually observed and reported for L-rhamnopyranose tetraacetate (27). Compound **7***a* is more dextrorotatory than **7***b*. The predominant formation of **7***a* may be rationalized in terms of the anomeric effect (26, 28). Therefore the structures of **7***a* and **7***b*, including the absolute configurations at C-6 and C-6′, are elucidated to be methyl (6*R*)-6-acetoxy-2,3-di-*O*-acetyl-4,6-*O*-[(6′*R*)-methyl-2′,3′,4′-tri-*O*-acetyl-6′-deoxy-β-D-galactopyranosid-6′-ylidene]-β-D-galactopyranoside and its (6*S*) isomer, respectively.

The ratio of the yields of **6** and **7** was a function of the enzymic reaction time, and prolonged reaction time caused the preponderant formation of the dimer **7**.

Since the structures of all isolable products were elucidated, and the H-6 signals of these products in their ^1H nmr spectra turned out to be diagnostically useful for our study, the galactose oxidase reaction with deuterated substrates was investigated.

[6]It is recognized that our coupling constants, obtained by first-order analysis, given in Table 2, and hence the rotamer populations in Table 2, are only approximately correct. The true coupling constants can only be obtained by full analysis including all spins involved.

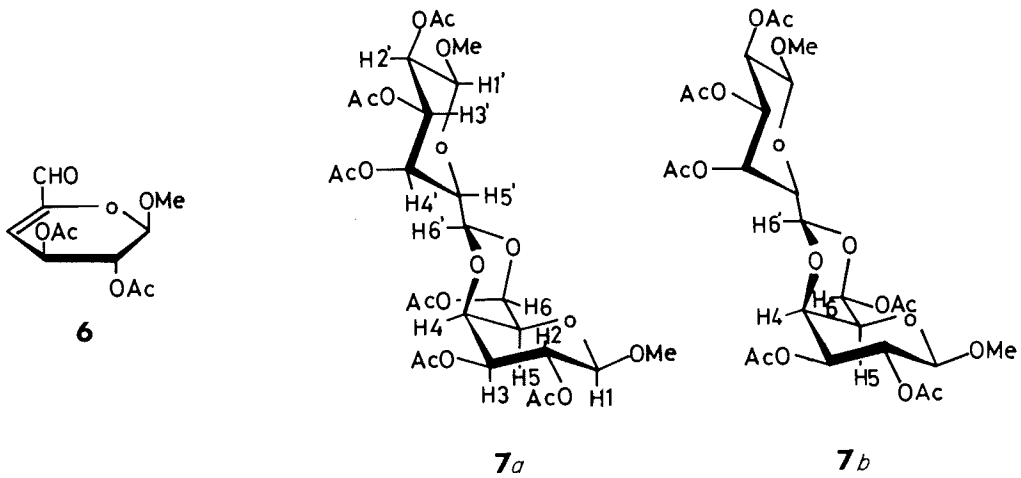
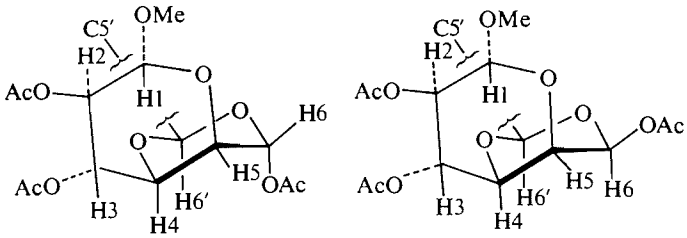


FIG. 5. Structures of the products.

TABLE 3. Proton chemical shifts of **7***a* and **7***b*



Proton	Shift (ppm)[a]		
	7*a*	**7***b*	Δ **7***b* − **7***a*
H1	(4.39)[b]	4.36–4.37	−0.02 to −0.03
H2	5.05–5.25 (5.18)[b]	5.05–5.25	±0
H3	4.77	4.75	−0.02
H4	(4.40)[b]	4.21	−0.19
H5	3.37 (3.39)[b]	3.66	+0.29
H6	6.22 (6.22)[b]	5.91	−0.31
H1′	(4.37)[b]	4.37	±0
H2′	5.27	5.25	−0.02
H3′	5.05–5.25 (5.08)[b]	5.05–5.25	±0
H4′	5.54 (5.55)[b]	5.54	−0.01
H5′	3.78 (3.81)[b]	3.85	+0.07 to +0.04
H6′	5.03	4.80	−0.21
OAc	1.9–2.2 (six OAc)	1.9–2.2 (six OAc)	±0

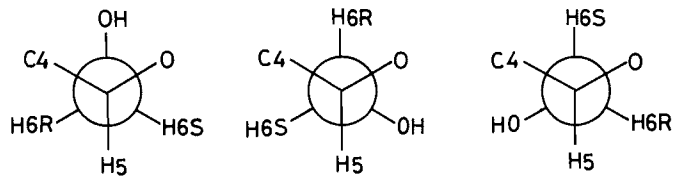
[a]Measured at 100 MHz in $CDCl_3$ at 296 K relative to internal TMS (0.000 ppm).
[b]Values quoted from ref. 26.

The relative rates of oxidation of these four substrates were first examined. Although the detailed kinetics (6) were not examined, under the same conditions it took 1.5 h for **3***R*, 3.0 h for **3**, 4.5 h for **3***S*, and more than 30 h for **3***DD* to be completely oxidized, judged by the disappearance of the starting material on tlc. Here it is interesting to note that the most favorable substrate for the enzyme is not the normal galactoside **3** but the (6*R*)-(6-$^2H_1$)-galactoside **3***R*.

The products from (6,6-$^2H_2$)-galactoside **3***DD* completely retained a deuterium atom at C-6, which indicated no exchange of hydrogen atom with the reaction medium during the reactions. The products **6**, **7***a*, and **7***b* from **3***R* contained 1% of proton at C-6, judged by both ^1H nmr and mass spectra. The results indicated the complete stereospecific removal of the *pro-S* hydrogen atom from the substrate. The products from (6*S*)-(6-$^2H_1$)-galactoside **3***S* (99% pure) retained about 80% of one proton at C-6 (loss of about 20% proton). The result indicated that the *pro-S* stereospecificity of the enzyme was substantially retained for the substrate but some of the *pro-R* hydrogen atoms were removed.

The above results can be rationalized as follows: There are two mechanisms by which the enzyme can oxidize the substrate; one is a very efficient *pro-S* hydrogen specific removal mechanism and the second is a far less efficient non-specific or *pro-R* hydrogen specific removal mechanism, which does not work detectably for the *pro-R* deuterium because of its inefficiency (see isotope effect (6) and the reaction rates (*vide supra*)).

The difference between the stereospecificity of the galactose oxidase oxidations of Perlin (6) and ourselves might be attributed to the difference in the origins of the enzymes.



Here, the plausible conformation of D-galactose residues in the binding site of D-galactose oxidase should be discussed based on the above results. Although Fig. 1 explains the selective removal of the *pro-S* 6-hydrogen atom, the *tg* conformation is somewhat questionable because of the following: (1) The preferred rotamer of D-galactose is not *tg* but *gt*. (2) In the *tg* conformation, the more acidic hydrogen atom of 4-OH is accessible to the imidazole group, resulting in the deactivation of the imidazole group (Fig. 6). (3) Since the assignments of H6*proR* and H6*proS* have been mistaken (4, 8), the proton that has been assigned as axial by ^{13}C and ^{19}F nmr relaxation experiments (3) would not be H6*proS* but H6*proR*. If this is the case, these nmr experiments indicate also that the preferred rotamer of D-galactose is *gt*.

It has been known that 4-deoxy-D-galactose (4-deoxy-D-glucose) is a fairly good substrate for the enzyme, indicating

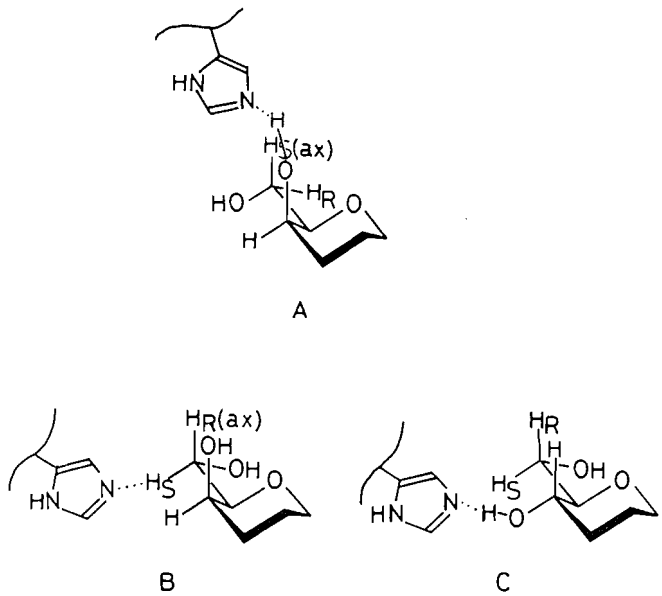


FIG. 6. Possible conformation of hexopyranose residues in the binding site of D-galactose oxidase; A, *tg* rotamer of D-galactose; B, *gt* rotamer of D-galactose; and C, *gt* rotamer of D-glucose.

that the 4-OH of D-galactose is not an important factor in the sugar being a substrate, but D-glucose is not a substrate at all, indicating that the 4-OH of D-glucose (equatorial 4-OH) should not be present if the sugar is to be the substrate of the enzyme (3). If the hydroxymethyl groups of the hexopyranoses are oxidized in the *gt* conformation as shown in Fig. 6, the above problems are solved, and the fact that D-glucose is not the substrate will be rationalized as follows (of course other steric factors have to be taken into account): In the *gt* conformation the more acidic hydrogen atom of the 4-OH of D-glucose, which is in 1,3-*syn*-diaxial relation with the *pro-S* 6-hydrogen, is accessible to the imidazole and reacts much faster than the *pro-S* 6-hydrogen, resulting in no oxidation of the hydroxymethyl group. Therefore, the *gt* conformation is recommended as a more plausible rotamer of D-galactose than *tg* in the binding sites of the enzyme.

Our results show that chirally pure deuterated sugars are very useful for both ^{1}H nmr studies of carbohydrates and stereochemical studies of enzymatic reactions. Studies along this line are being undertaken in our laboratory.

Experimental

Melting points were taken with a Yanako Model P hotplate apparatus and were uncorrected. The ^{1}H nmr spectra were recorded on JEOL JNM-FX 100 or JEOL FX 400 instruments with TMS as an internal standard in $CDCl_3$, or with 3-(trimethylsilyl)propanesulfonic acid sodium salt as an internal standard in D_2O. Direct mass spectra were recorded on a Hitachi M-52 spectrometer with the ionizing potential at 70 eV. Specific rotations were measured on a Jasco ORD/UV at 589 nm. Merck silica gel 60 (Art. 7734) was used for column chromatography and Merck silica gel 60 F_{254} (Art. 5548) was used for both preparative and analytical thin-layer chromatographies (tlc).

Materials

The substrates **3***R* and **3***S* were prepared from the corresponding (6*R*)-(6-$^2H_1$)- and (6*S*)-(6-$^2H_1$)-D-galactoses (12, 13), respectively, according to conventional methods (6). D-Galactose oxidase (EC 1.1.3.9.) Type V from *Dactylium dendroides* and catalase from bovine liver were purchased from Sigma Chemical Company.

*1,6-Anhydro-2,3,4-tri-O-benzoyl-D-talopyranose (**4**)*

To a solution of 1,6-anhydrotalopyranose (20) (1.7 g) in dry pyridine (50 mL) was added benzoyl chloride (10 g). After the mixture had been stirred for 12 h at room temperature, water (50 mL) was added to the reaction mixture and it was stirred for 2 h. The mixture was extracted with $CHCl_3$ (25 mL × 3) and the $CHCl_3$ layer was successively washed with water, saturated $NaHCO_3$, and water and then dried over $MgSO_4$. Evaporation of the solvent gave a syrup, which was crystallized from ethyl ether – hexane to give **4** (4.5 g, 90%); mp 178°C; $[\alpha]_D^{21}$ −58° (*c* 0.1, $CHCl_3$).

*Methyl 2,3-O-acetyl-4,6-O-benzylidene-β-D-galactopyranoside (**5**) and its (6S)-(6-$^2H_1$) derivative (**5S**)*

A solution of **3** (500 mg, 2.6 mmol), benzaldehyde dimethyl acetal (1 g, 6.6 mmol), and *p*-toluenesulfonic acid (5 mg) in *N*,*N*-dimethylformamide (20 mL) was stirred for 1 h at 55–60°C under reduced pressure (water pump) to remove methanol from the reaction mixture. To the stirred solution was added anhydrous sodium acetate (100 mg), pyridine (20 mL), and acetic anhydride (5 mL), and the suspension was stirred overnight at room temperature. The tlc analysis of the reaction mixture indicated the formation of two products (R_f 7.4, minor, and R_f 6.8, main; benzene/ethyl acetate 8:1, v/v). The mixture was evaporated *in vacuo* and the residue was chromatographed on a silica gel column with a mixture of benzene and ethyl acetate (50:1, v/v) to give methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranoside **5** (480 mg, 50%), mp 148–150°C; $[\alpha]_D^{23}$ +65.2° (*c* 1.1, $CHCl_3$). ^{1}H nmr (δ(ppm, $CDCl_3$)): 4.44 (1H, d, 8.0 Hz, H-1), 5.39 (1H, dd, 8.0 and 10.4 Hz, H-2), 4.96 (1H, dd, 3.6 and 10.4 Hz, H-3), 4.38 (1H, br d, ≅3.6 Hz, H-4), 3.5 (1H, br s, H-5), 4.07 (1H, dd, 1.7 and 12.4 Hz, H6*proR*), 4.34 (1H, dd, 1.7 and 12.4 Hz, H6*proS*), 3.52 (3H, s, OMe), 5.51 (1H, s, Ph-CH), 2.07 (6H, s, OAc). *Anal.* calcd. for $C_{18}H_{23}O_8$: C 58.84, H 6.31; found: C 58.71, H 6.13. Also obtained was methyl 2,6-di-*O*-acetyl-3,4-*O*-benzylidene-β-D-galactopyranoside (70 mg, 7%), mp 132–143°C; $[\alpha]_D^{23}$ +16.8° (*c* 0.8, $CHCl_3$). ^{1}H nmr (δ(ppm, $CDCl_3$)): 4.35 (1H, d, 7.7 Hz, H-1), 5.11 (1H, dd, 5.5 and 7.7 Hz, H-2), 4.51 (1H, dd, 5.5 and 6.5 Hz, H-3), 4.22 (1H, dd, 2.0 and 6.5 Hz, H-4), 3.98 (1H, q, 2.0, 5.6 and 7.7 Hz, H-5), ≅4.4 (2H, H-6,6′). *Anal.* calcd. for $C_{18}H_{23}O_8$: C 58.84, H 6.31; found: C 59.10, H 6.22.

The (6*S*)-(6-$^2H_1$) derivative (**5***S*) of **5** was obtained by the same procedures as described for **5**. The physical data of **5***S* are essentially the same as those of **5** except for the chemical shifts of H6*proR* (δ 4.06) and H5 (δ 3.49).

Oxidation of methyl β-D-galactopyranosides with D-galactose oxidase

In a typical experiment, the substrate (40 mg) was dissolved in phosphate buffer (4 mL:0.1 *M*, pH 6.0) and incubated with D-galactose oxidase (5 mg, 350 units) and catalase (5 mg) at 28°C. The reaction was monitored by tlc (*n*-propanol:acetic acid:water 6:1:2, v/v). The reaction was stopped when the tlc showed the complete disappearance of the substrate (R_f 0.58) and the formation of main product (R_f 0.69) (it took 1.5 h for **3***R*, 3.0 h for **3**, 4.5 h for **3***S*, and more than 30 h, i.e., usually did not complete, for **3***DD*). A mixture of Amerlite IR-120 (H^+) and Dowex-1 (bicarbonate) was added to the digest with stirring, and it then was lyophilized. The product (40 mg) was treated with pyridine (1 mL) and acetic anhydride (0.5 mL) at room temperature overnight, the reaction mixture was concentrated, and the syrupy residue was chromatographed on a column of silica gel (5 g). Elution with a mixture of benzene and ethyl acetate (10:1, v/v) afforded methyl 2,3-di-*O*-acetyl-α-L-*threo*-4-deoxyhex-4-eno-dialdopyranoside **6** as a syrup (4 mg) (R_f 0.75, benzene:ethyl acetate 10:1, v/v). The subsequent elution with a mixture of benzene and ethyl acetate (3:1, v/v) afforded a mixture of **7***a* and **7***b* (18 mg). The mixture was separated by preparative tlc using a mixture of $CHCl_3$ and MeOH (70:1, v/v, three developments) to give a crystalline **7***a* (9 mg), mp 204–205°C, $[\alpha]_D^{22}$ +38.4° (*c* 0.07, EtOH) (lit. (26) mp 206–208°C; $[\alpha]_D^{22}$ +41.6° (*c* 2.1, $CHCl_3$)). *Anal.* calcd. for $C_{26}H_{36}O_8$: C 49.00, H 5.70; found: C 49.08, H 5.95; and **7***b* (3 mg), mp 130–131°C; $[\alpha]_D^{22}$ ±0° (*c* 0.04, EtOH). *Anal.* calcd. for $C_{26}H_{36}O_{18}$: C 49.00, H 5.70; found: C 49.25, H 6.00.

The (6,6′-$^2H_2$) derivative of **7***a*, obtained by oxidation of **3***DD*, had

mp 208–210°C; $[\alpha]_D^{22}$ +45.5° (*c* 0.05, EtOH) (lit. (26) mp 210–211°C; $[\alpha]_D^{22}$ +47° (*c* 0.4, $CHCl_3$)).

Oxidation of **3** R *and* **3** S

Oxidation of **3***R* gave the same products as with **3***DD*. Oxidation of **3***S* gave mixtures of **6** and the (6-$^2H_1$) derivative of **6**, and of **7***a* and the (6,6′-$^2H_2$) derivative of **7***a* in ratios ca. 80:20 judged by both ^{1}H nmr and mass spectroscopies.

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