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Stereochemistry of Dehydrogenation by D-Galactose Oxidase[1]

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The stereochemistry of dehydrogenation of the primary carbinol group of D-galactose by D-galactose oxidase has been determined. Using D-galactose-6-*d* and methyl β-D-galactopyranoside-6-*d*, it has been established that the reaction involves removal of the *pro-S* 6-hydrogen atom. This conclusion is based on product analysis, and on the relative impact of the deuterium isotope effect on oxidation rates of substrates having different *R*:*S* deuteration patterns. The absolute configurations at C-6 of these substrates have been determined by selective chemical transformations to products of known configuration. The rotational conformation of the 6-carbinol group of D-galactose and its possible relationship to the specificity of the enzyme are discussed, as well as the stereochemistry of reductive deuteration of *aldehydo* sugar derivatives.

On a déterminé la stéréochimie de déshydrogénation du groupement carbinol primaire du D-galactose par le D-galactose oxydase. Il a été établi, en utilisant le D-galactose-6-*d* et le méthyl β-D-galactopyranoside-6-*d*, que la réaction implique le départ de l'atome d'hydrogène-6 *pro-S*. Cette conclusion se base sur le produit d'analyse et sur l'impact relatif de l'effet de l'isotope deutérium sur les vitesses d'oxydation des substrats ayant des modèles différents de deutération *R*:*S*. Les configurations absolues en C-6 de ces substrats ont été déterminées par des transformations chimiques sélectives en produits de configuration connue. La conformation rotationnelle des groupements carbinole-6 du D-galactose et sa relation possible avec la spécificité de l'enzyme sont discutées, de même que la stéréochimie de deutération réductive des dérivés *aldéhydo* des sucres.

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D-Galactose oxidase from *Polyporus circinatus* (1, 2) (D-galactose oxireductase) catalyzes the reaction

$$\text{D-Galactose} + O_2 \rightarrow \text{DL-Galactohexodialdose} + H_2O_2$$

that is, it converts the primary carbinol group of D-galactose to an aldehyde. D-Galactopyranosides and unsubstituted D-galactopyranosyl residues of various oligo and polysaccharides also are oxidized by the enzyme (2–4), but attack is inhibited or retarded by the introduction of substituents at secondary positions of the D-galactopyranosyl moiety (5).

It was assumed *a priori* that D-galactose oxidase must effect a stereospecific removal of one of the C-6 hydrogen atoms, as is true for several dehydrogenases that have been examined (6–9), and this was subsequently found to be the case. To examine the precise stereochemistry involved, the well-established approach of utilizing a partially deuterated substrate of known configuration (6–11) was adopted.

The chirality of the 6-carbinol group may be described *e.g.*, as for methyl β-D-galactopyranoside (**1**), in terms of replacement of a hydrogen atom by deuterium (9, 12, 13). That is, H_S is designated as the *pro-S* hydrogen (deuterium in this position would confer an *S* configuration upon the carbinol group as in **2**) and H_R, the *pro-R* hydrogen as in the *R* diastereomer **3**. From the correlations of absolute configuration described below, it is concluded that the *pro-S* hydrogen is removed specifically by D-galactose oxidase.

Samples of methyl β-D-galactopyranoside[4] monodeuterated at position-6 (**1***a* and *b*) were prepared as substrates for this study by reaction sequences involving, as the key step, reduction of 1,2:3,4-di-*O*-isopropylidene-α-D-galactohexodialdose-1,5-pyranose (**4***a*) (14) with sodium borodeuteride (for **1***a*), and reduction of the dialdose-6-*d* **4***b* with sodium borohydride (for **1***b*)[5] (Scheme 1). Analysis of **1***a* (below) showed that the distribution of deuterium in the 6-car-

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[4]This glycoside, which is one of the most reactive substrates of D-galactose oxidase (3), proved to be more satisfactory in some respects than D-galactose itself. However, the stereochemistry of the reaction with D-galactose was found to be the same, as shown below.

[5]An attempt to achieve stereospecific deuteration by reduction with yeast (15) was unsuccessful: although the yeast culture rapidly reduced benzaldehyde, it had no noticeable effect on the deuterated *aldehydo* sugar derivative **4***b*.

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ERRATUM

3429

Erratum: Stereochemistry of Dehydrogenation by *D*-Galactose Oxidase

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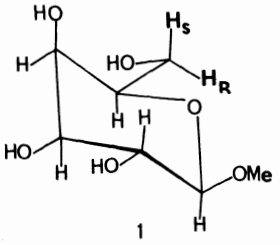
On p. 3429, right-hand column, lines 5 and 6, the numbers **2** and **3** should be interchanged, *i.e.* the lines should read:

"... upon the carbinol group as in **3**) and H_R, the *pro-R* hydrogen as in the *R* diastereomer **2**. From ..."

On. p. 3430, left-hand column, lines 10 and 11, Scheme 1 should read Scheme 2, *i.e.*:

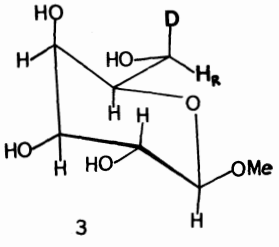
"... configurational correlations illustrated on Scheme 2."

FOR ERRATA SEE
Vol. 50 1972 P. 768

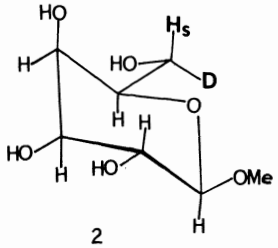


1

a 2+3 (3·6:1)
b 2+3 (1:3)



3



2

binol group corresponded to a mixture of 3.6 parts of the *R*-diastereomer and 1 part of the *S*-diastereomer; *i.e.*, 3.6 of **2**: 1 of **3**. In **1***b*, the distribution of isotope constituted a diastereoisomeric mixture of 1 part of (*R*)(**2**) to 3.0 parts of (*S*)(**3**). The stereochemistry of these reductions is considered further below.

These stereochemical descriptions of the C-6 chiral centers in **1***a* and *b* were obtained *via* the configurational correlations illustrated in Scheme 1.[6] D-Galactose-6-*d* (**5***a* or *b*), obtained in the course of synthesis of **1***a* or *b*, respectively, was degraded to D-threose-4-*d* by oxidation with lead tetraacetate (16) and the tetrose, then acetylated to give crystalline tri-*O*-acetyl-α-D-threofuranose-4-*d* (**6***a* or *b*) respectively. The absolute configuration at the 4-position of **6** was then deduced by reference to tri-*O*-acetyl-α-D-threofuranose-4-*d* (**6***c*) prepared from D-xylose-5-*d* of known absolute configuration (11). This latter sequence (Scheme 2) comprised *O*-benzoylation of 1,2-*O*-isopropylidene-α-D-xylofuranose-5-*d*, hydrolysis, lead tetraacetate oxidation of 3,5-di-*O*-benzoyl-D-xylofuranose-5-*d*, and finally, conversion of the latter to **6***c*. A comparison of the isotope distribution at C-4 of **6***a*, *b*, and *c* was readily carried out by measuring the relative intensities of the well-separated H-4 signals in the p.m.r. spectrum of each of these products (Fig. 1). This showed that **6***a* and *c* are virtually indistinguishable but strikingly different from **6***b*. Since the configuration at C-5 of the D-xylose-5-*d* utilized as a reference point is predominantly *R*[7] (11), it follows that the configuration of D-galactose-6-*d* (**5***a*) also is predominantly *R*, and hence **5***b* predominantly *S*, as indicated above.

Glycosides **1***a* and *b* having been characterized, they were then used as substrates for D-galactose oxidase. The 6-aldehydo products (**7***a* and *b*, respectively) of these oxidations were acetylated in pyridine, with concomitant β-elimination, which afforded the known (18) unsaturated aldehyde, *i.e.*, methyl 2,3-di-*O*-acetyl-α-L-*threo*-4-deoxy-hex-4-enodialdopyranoside-6-*d* (**8***a* and *b* respectively). Measurements of the residual 6-deuterium and 6-protium contents of **8** and its dinitrophenylhydrazone by mass spectrometry and p.m.r. spectroscopy concurred in showing that the enzymatic dehydrogenation of **1***a* had involved loss of 80% of a 6-hydrogen present originally, whereas in **1***b* 75% of the corresponding 6-hydrogen remained. That is, in both instances the results demonstrate a specific removal of the *pro-S* 6-hydrogen atom.

This same stereospecificity for D-galactose oxidase has been deduced by comparing oxidation rates for D-galactose, D-galactose-6-*d* (**5***a* and *b*), and D-galactose-6,6′-d_2 (Table 1). Firstly, the relative rates for D-galactose and the dideuterio compound show that the dehydrogenation is subject to a kinetic isotope effect of about 7.7. Accordingly, **5***a* and *b* show intermediate, and substantially different, oxidation rates that reflect the relative proportions of hydrogen and deuterium removed in the process. Moreover, there is good agreement between the observed rates and those calculated, indicating that there is no substantial secondary kinetic isotope effect. Since **5***b* experiences a slower rate than **5***a*, and possesses a correspondingly higher proportion of the *S*-diastereomer, these results also demonstrate that the oxidative attack on D-galactose

[6]It is assumed that in all of these reactions the new chiral centers generated within the primary carbinol group by deuteration are unaffected.

[7]D-Xylose-5-*d* prepared in the current (and also in an earlier (17)) study contains a slightly higher preponderance of the *R* diastereomer than does D-xylose-5-*d* described initially by Lemieux and Howard (11); the current procedure differs in using sodium borodeuteride – water in place of lithium aluminum deuteride – ether.

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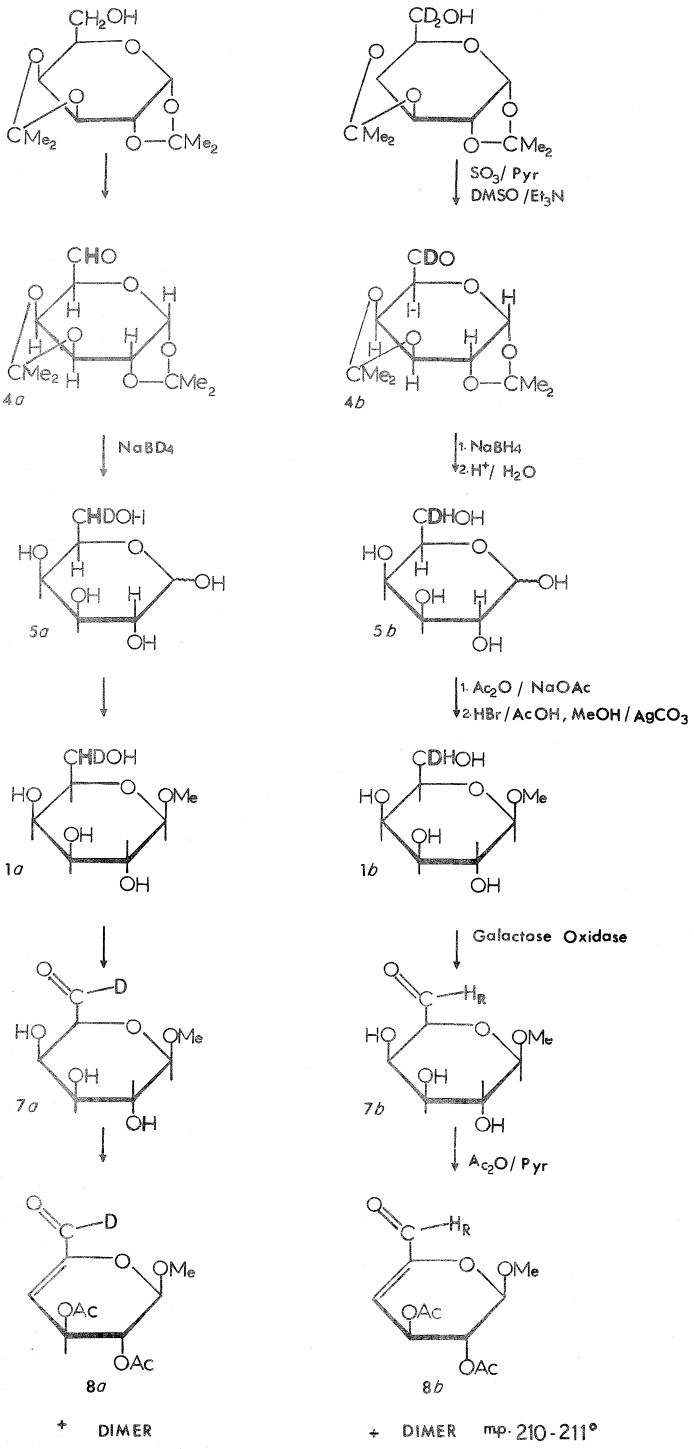

SCHEME 1. Synthesis of methyl β-D-galactopyranoside-6-*d* (1*a* and *b*) and examination of its oxidation by D-galactose oxidase.

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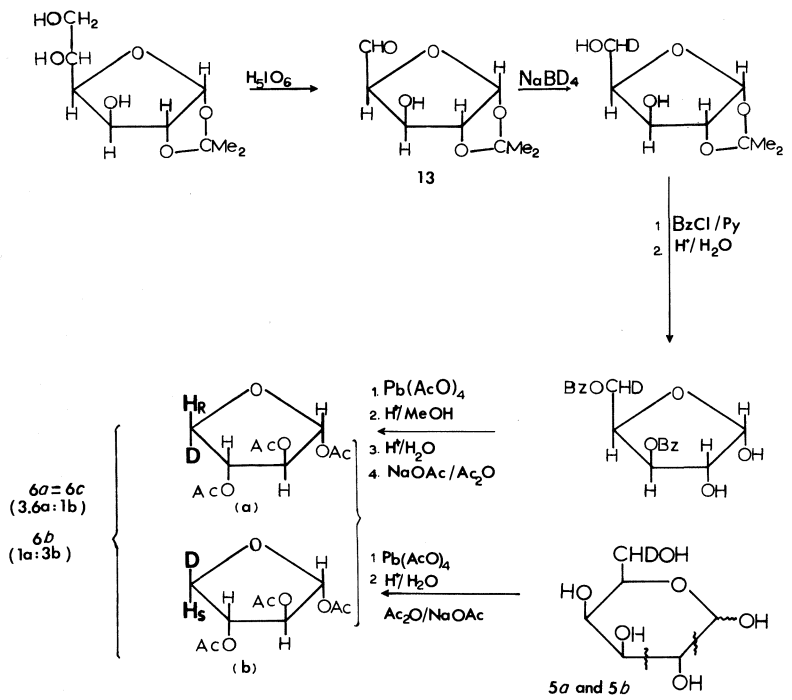

SCHEME 2. Sequence of reactions used in determining the absolute configuration of the 6-carbinol group in methyl β-D-galactopyranoside-6-*d* (**1***a* and *b*).

involves stereospecific abstraction of the *pro-S* 6-hydrogen atom.

Not only is the axial 4-hydroxyl group of D-galactose essential to the enzymatic oxidation, but substitution of its H atom (with methyl or glycosyl (5)) inhibits the reaction. By contrast, similar blocking of the 2- or 3-hydroxyl group has only a retarding effect on the rate, and modification at the 1-position influences rate even less. Evidence from the p.m.r. spectra of D-galactopyranose peracetates (19, 20) and spectral information provided below, suggests that a favored rotational conformation of the 6-carbinol group is one in which the *pro-S* hydrogen and the 4-hydroxyl group stand in a *cis*-1,3 relationship with respect to each other, as illustrated in **1**. It may be pertinent that in such an arrangement, the approaching enzyme would find the specificity-determining 4-hydroxyl group, the *pro-S* 6-hydrogen that is to be removed and the 6-hydroxyl group all in close spatial proximity to each other on the same face of the D-galactose substrate.[8] Perhaps an orientational relationship such as this accounts for the contrasting action of D-galactose oxidase with respect to ethanol dehydrogenase (from yeast (6) and liver (9)) or glycolic acid dehydrogenase (from plants (8)). That is, these latter enzymes remove H_R rather than H_S from the primary carbinol group.

Some information about how the 6-carbinol group of D-galactose derivatives is oriented has been deduced from H-5, H-6 coupling constants (19, 20): the two observed values of 7 and 5.5–6.0 Hz have been attributed to the *trans*–diaxial and *gauche* arrangements, respectively, of **9** or **10**. Rotamer **11** is unfavorable because it would involve eclipsing between OH-6 and axial OH-4 (19, 20). Examination of selectively deuterated acetates **1***a* and *b* at 220 MHz (Fig. 2) shows clearly that the larger spacing (7.2 Hz) arises from coupling between H-5 and H_S-6 (*i.e.*, as in **1** and **9**, though not as in **10**); at this higher frequency, the H-5, H_R-6 spacing is 6.3 Hz. These data may be interpreted most directly by assuming that in by far its most favorable orientation, the 6-carbinol group is close to **9** but rotated about 25° clockwise; which, incidentally, minimizes the O-4, H_S-6 eclipsing interaction, as found in **1**.

[8]However, no information is as yet available concerning the geometry of the active site of D-galactose oxidase, and the "favorableness" of such an arrangement of groups cannot be assessed.

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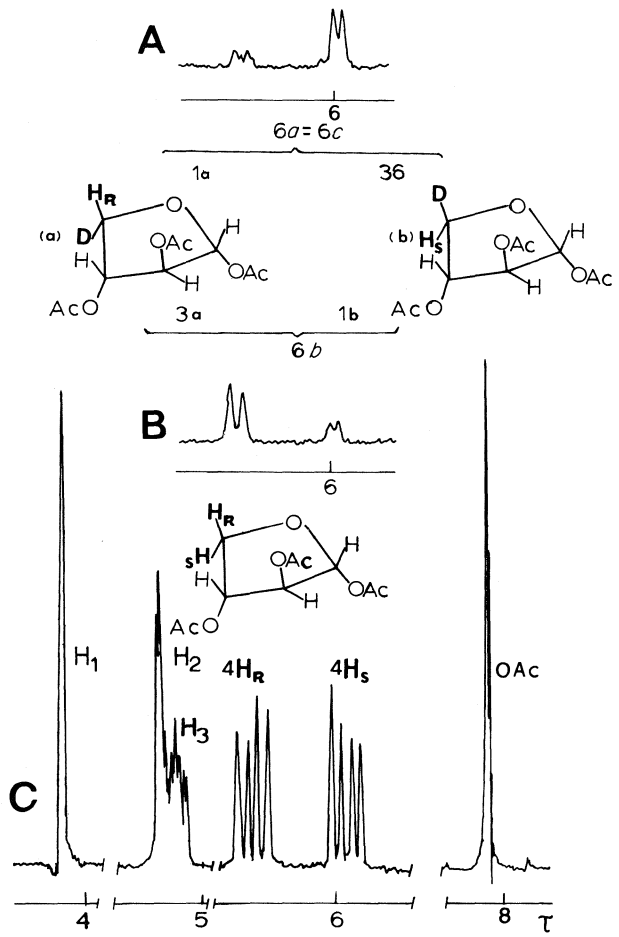


FIG. 1. The p.m.r. spectra at 100 MHz (in deuterochloroform), of 1,2,3-tri-*O*-acetyl-α-D-threofuranose and partially 4-deuterated specimens of this tetrose derivative (**6***a* and *b*); (*A*) H_4 signals of **6***a* and *c*, (*B*) H_4 signals of **6***b*, (*C*) the total spectrum of the undeuterated compound, with the acetoxyl proton signal (OAc) being recorded at about 2/3 relative intensity. The conformations depicted are taken to be approximately representative of the spectral information (unpublished results).

TABLE 1. Rates of oxidation of D-galactose and deuterated D-galactoses by D-galactose oxidase*

Substrate	Rate† (observed)	Contribution due to C—H	Contribution due to C—D	Rate (calculated)
D-Galactose	0.77	—	—	—
D-Galactose-6-*d* (3.6*R*:1*S*)	0.66	0.60	0.02	0.62
D-Galactose-6-*d* (1*R*:3*S*)	0.28	0.19	0.08	0.27
D-Galactose-6,6′-d_2	0.10	—	—	—

*Colorimetric assay at 420 nm (Experimental).
†Slope of the plot of optical density *vs.* reaction time from 2 to 12 min; during this initial period each plot was virtually a straight line.

According to the Karplus curve (21), the expected couplings then are $J_{5,6(S)}$ 7.5 and $J_{5,6(R)}$ 6.0 Hz, which are close to the splittings observed. Nevertheless, it is reasonable to expect that a mixed population of rotamers exists, in which energy differences are relatively small. In this event, the large coupling shown to take place between H-5 and H_S-6 indicates that **9** should be an important contributor to the rotational itinerary.

The stereochemistry of hydride reduction of the

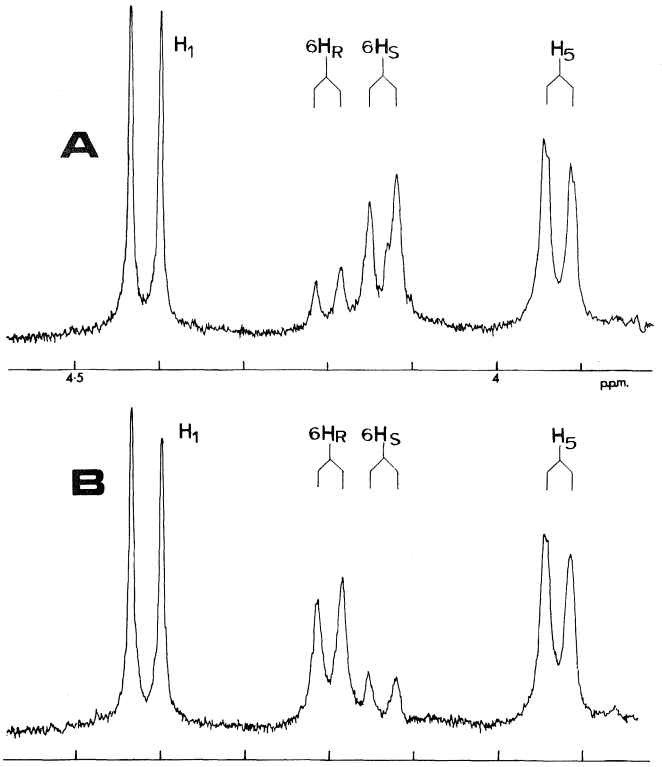
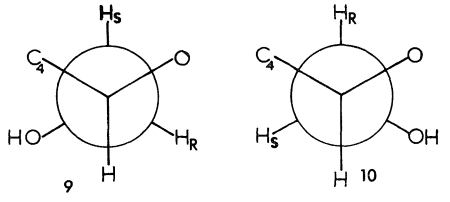
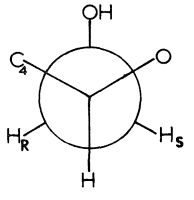
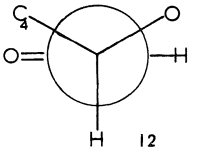


FIG. 2. Partial p.m.r. spectrum at 220 MHz (in deuterochloroform) of methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside-6-*d* (**1***a* (*A*) and *b* (*B*)).

6-*aldehydo* compound **4** merits comment. A predominance of the *R* carbinol product, as found, is accounted for most readily by a topology in which the hydride ion approaches from the least hindered, *re*-face (12, 22–24), and by assuming (24, 25) that the stereochemistry of the starting material and product are closely similar. According to the p.m.r. spectrum of **4**, which shows that H-6 is not coupled with H-5, the C=O group appears to be oriented preferably *gauche* with respect to the C_4—C_5 bond (as in **12**); this deviates somewhat from the Karabatsos model (24) in which the C=O group would be eclipsed with one of the C-5 substituents. From the alternative possibility, *i.e.*, C=O *gauche* with respect to the C_5—O_5 bond, a predominantly *S* carbinol should be formed, contrary to the result obtained. Conceivably, however, O-5 might participate in stabilizing the transition complex, as in the "cyclic model" of Cram *et al.* (22, 23), but then could lead to a predominantly *R* product only if attack from the *more* hindered *si*-face were facilitated in the complex. The slightly lower







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degree of stereoselectivity in the reduction of **4***b* than of **4***a* is attributable possibly to a slower transfer of a deuteride (26, 27), than of a hydride, anion with a concomitant increase in 'the randomness of the reaction. Reduction of the 5-*aldehydo* pentose derivative **13** with aqueous borohydride presumably involves a topology similar to that for **4**, because of the similar diastereomeric makeup of both products, despite the fact that **13** exists as a *gem* diol (in deuterium oxide (17)). More directly comparable with **4** as an *aldehydo* compound is the 3-*O*-benzyl derivative of **13** which, when reduced with lithium aluminum deuteride, also gives rise predominantly to the corresponding *R* carbinol (11), as already noted.

Experimental

Melting points were taken with a Fisher–Johns hot-plate apparatus and are uncorrected. The i.r. spectra were recorded using KBr discs or in solution with a Unicam Sp-200 G spectrometer. Electronic spectra were recorded with a Unicam Ultraviolet spectrometer. Mass spectra were recorded with a double-focusing AEI spectrometer; the energy of ionization was 70 eV. The n.m.r. spectra were recorded with a Varian 100 MHz HA-100 spectrometer using an internal TMS lock signal, or a Varian A60 spectrometer. Spectra at 220 MHz were provided by the Canadian 220 MHz NMR Centre, Sheridan Park, Ontario.

Column chromatography was performed using cellulose powder for aqueous systems and silica gel (Macherey, Nagel and Co.) for non-aqueous systems. The t.l.c. plates were prepared from silica gel G according to Stahl and were dried in a 100 °C oven for 1 h, and cooled in a vacuum desiccator. Descending chromatography on Whatman No. 1 was used for all paper chromatography work; reducing sugars were detected with silver nitrate (28) or with aniline oxalate (29). Solvents for paper chromatograms were butanol:ethanol:water (4:1:5 upper layer) (28) or *n*-propanol:acetic acid:water (6:1:2) (3), unless otherwise specified. Visualization of t.l.c. plates was effected with 5% concentrated sulfuric acid in ethanol or 2,4-dinitrophenylhydrazine in phosphoric acid and ethanol; with both reagents, carbohydrates and derivatives showed up as dark spots on a lighter background; α, β-unsaturated aldehydes and benzoates were also visualized with a u.v. lamp.

*1,2:3,4-Di-O-isopropylidene-α-D-galactohexodialdose-1,5-pyranose (**4a**)*

1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranose (prepared by de-*O*-acetylation of the crystalline 6-acetate, m.p. 109–110° (30)) was oxidized with methyl sulfoxide in the presence of triethylamine – sulfur trioxide as described previously (14). The diketal (1.5 g) was dissolved in methylsulfoxide (10 ml), triethylamine (3 ml) was then added, followed by a solution of sulfur trioxide – pyridine (2.8 g) in methylsulfoxide (10 ml). On shaking, the mixture rapidly became warm and darkened (reaction was complete within a few minutes, as indicated by t.l.c.) (benzene:ether (1:1)). It was then cooled in acetone–CO_2 and slowly diluted with chloroform (200 ml), then washed successively with 2 *N* hydrochloric acid, water, dilute sodium bicarbonate, water (2 ×). The dried chloroform solution was concentrated to a syrup (1.0 g), which was chromatographed on a column of silica gel with benzene:ether (1:1) as eluant to separate the aldehyde **4** (1.0 g) from a small amount (50 mg) of accompanying 1,2:3,4-di-*O*-isopropylidene-6-*O*-(methylthio)-methyl-α-D-galactopyranose.

6-O-Acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6-d (3.6 R : 1 S)

To a stirred solution of 1,2:3,4-di-*O*-isopropylidene-α-D-galactohexodialdose-1,5-pyranose (**4***a*) (1.0 g) in ice-cold ethanol:water (1:1(15 ml), a solution of sodium borodeuteride (0.2 g) in ice water (5 ml) was slowly added, and stirring was continued for 30 min (reduction complete as shown by t.l.c.). Excess of Amberlite IR-120 resin was added, the solution was evaporated to dryness, and boric acid was removed by repeatedly dissolving the residue in methanol, followed by concentration to dryness. The reduction product (0.9 g) was acetylated with acetic anhydride (2.5 ml) in pyridine (5 ml), affording crystalline 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose-6-*d* (0.7 g); m.p. 107–108°.

*Methyl β-D-Galactopyranoside-6-d (**1a**)*

1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranose-6-*d* (prepared from the above 6-acetate) (1.0 g) was dissolved in 0.1 *N* sulfuric acid (40 ml), the solution was heated at 95° for 45 min, cooled, and neutralized with barium carbonate. Filtration was facilitated by use of Celite, the filtrate was treated with Amberlite IR-120 resin, then concentrated, and the residue was crystallized with cold methanol; yield of α-D-galactose-6-*d* (**5***a*) 0.5 g, m.p. 164–166°. The sugar (1.0 g) was converted successively by well-established procedures (31–33) to 1,2,3,4-6-penta-*O*-acetyl-β-D-galactopyranose-6-*d*, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-6-*d*-bromide, methyl tetra-*O*-acetyl-α-D-galactopyranoside-6-*d* and, finally, to methyl β-D-galactopyranoside-6-*d* (**1***a*) m.p. 176–177° (0.3 g).

6-O-Acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6,6′-d_2

1,2:3,4-Di-*O*-isopropylidene-α-D-galacturonic acid (34) (m.p. 158.5–159.5°) was converted to the methyl ester with diazomethane. The ester (7.5 g) in ether (100 ml) was reduced with lithium aluminum deuteride (1.0 g) dissolved in ether (150 ml), the deuteride being added over a period of 30 min with good stirring and under gentle reflux. Ethyl acetate (2 ml) and water (50 ml) were added in succession, followed by acidification with 2 *N* hydrochloric acid and dilution with water (100 ml). The aqueous layer was extracted with chloroform, and the combined chloroform and ether solutions (after washings with sodium bicarbonate and water) were dried and concentrated. Acetylation of the residue (6.1 g), as above, afforded 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose-6,6′-d_2 (6.5 g), m.p. 110°.

*Methyl β-D-Galactopyranoside-6-d (**1b**)*

1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranose-6,6′-d_2 (5.2 g) (prepared from the 6-acetate, previous section) was oxidized to the 6-deuterio dialdose (**4***b*) as

described for **4***a* above;[9] yield, 4.0 g. This syrupy aldehyde, in ethanol:water (1:1)(30 ml) was reduced at 10° with sodium borohydride (0.4 g), the product was purified by chromatography on silica gel (benzene:ether (1:1) as eluant) (yield, 2.8 g), and then acetylated with acetic anhydride – pyridine to give 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose-6-*d*; m.p. 109–110°. The latter was deacetylated and hydrolyzed, and the D-galactose-6-*d* (**5***b*) (0.9 g) obtained was converted via the acetobromo derivative, as described above, to methyl β-D-galactopyranoside-6-*d* (**1***b*) (0.3 g); m.p. 167°.

*1,2,3-Tri-O-acetyl-α-D-threofuranose-4-d (**6***a and b)*

D-Galactopyranose-6-*d* (**5***a* or *b*) was oxidized in acetic acid solution with 2 mol of lead tetraacetate per mole (16). The syrupy formate ester obtained was hydrolyzed, and the product acetylated with acetic anhydride – sodium acetate, yielding 1,2,3-tri-*O*-acetyl-α-D-threofuranose-4-*d* (**6***a*, m.p. 120–121°, $[\alpha]_D$ +35° ($CHCl_3$); or **6***b*, m.p, 120–121°, $[\alpha]_D$ +37° ($CHCl_3$)).

*1,2,3-Tri-O-acetyl-α-D-threofuranose-4-d (**6***c)*

1,2-*O*-Isopropylidene-α-D-xylofuranose-5-*d* (17) (6.0 g) was converted to 3,5-di-*O*-benzoyl-D-xylofuranose-5-*d*, using conditions for benzoylation and partial hydrolysis described by Baker and Schaub (35). The syrupy dibenzoate obtained (5.6 g), dissolved in acetic acid (300 ml), was oxidized for 1 h with a solution of lead tetraacetate (8.5 g) in acetic acid (250 ml), and after removal of most of the solvent the product was extracted into benzene; yield, 4.5 g of syrup. Methanolysis of this syrup was effected with methanol (200 ml) containing acetyl chloride (1.6 ml), under reflux for 18 h, the solution was neutralized with silver carbonate, concentrated, and the product was hydrolyzed with 0.1 *N* sulfuric acid (30 ml) at 95° for 1 h. The hydrolysate, after neutralization with barium carbonate and Amberlite IR-120 resin, was found by chromatography to consist mainly of threose; a small proportion of xylose was detected, as well as some other minor, unidentified, components. By column chromatography on cellulose (eluant: one-quarter saturated butanol), the threose was separated out (0.3 g) and then was acetylated to yield, finally, 1,2,3-tri-*O*-acetyl-α-D-threofuranose-4-*d* (**6***c*); m.p. 118–120°.

*Oxidation of Methyl β-D-Galactopyranoside-6-d (**1***a and b) with D-Galactose Oxidase*

In a typical experiment, the substrate (40 mg) was dissolved in phosphate buffer (4 ml; 0.2 *M*, p*H* 7.0) and incubated with D-galactose oxidase (5 mg, 125 units) and catalase (5 mg) at 37 °C for 3 h (t.l.c examination showed the presence of a major product (R_f 0.63 as compared with R_f 0.47 for the intact glycoside), and two minor products (R_f 0.75 and 0.46, respectively)). A mixture of Amberlite IR-120 (H^+) and Dowex-1 (bicarbonate) was added to the digest with stirring, the suspension was filtered, the filtrate concentrated, and then lyophilized. The product (36 mg) was treated with pyridine (0.8 ml) and acetic anhydride (0.4 ml) at room temperature for 18 h, the reaction mixture was concentrated, and the syrupy residue chromatographed on a column of silica gel (eluant, benzene:ether (9:1)). This afforded methyl 2,3-di-*O*-acetyl-α-L-*threo*-4-deoxy-hex-4-enodialdopyranoside (**8***a* or *b*)[10] (10 mg) followed by methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (3 mg from the undeuterated glycoside; 7 mg from **1***a*; 12 mg from **1***b*). The subsequent use of benzene:ether (1:4) caused elution of a third component which has been characterized (unpublished results), as a dimer originating from the aldehydic oxidation product having R_f 0.46.

2,4-Dinitrophenylhydrazone of Methyl 2,3-Di-O-acetyl-α-L-threo-4-deoxyhex-4-enodialdopyranoside

The α,β-unsaturated aldehyde (10 mg) was dissolved in ether (0.5 ml) and to this solution was added 2,4-dinitrophenylhydrazine (0.1 *M* in phosphoric acid – ethanol (36) (3–4 drops). Orange–yellow crystals which soon formed on standing, were washed with ethanol and ether; m.p. 148–152°, undepressed on admixture with a specimen prepared from an authentic sample of the α,β-unsaturated aldehyde (18). The n.m.r. and mass spectra of the two hydrazones were indistinguishable. A higher melting form of this hydrazone (m.p. 167°) which, however, is indistinguishable from the current product by n.m.r. and mass spectrometry also has been obtained (18).

Measurement of Reaction Rates for D-Galactose Oxidase

Kinetic measurements were carried out using "Galactostat" (Worthington). The "Galactostat" solution consisted of D-galactose oxidase (20 units), peroxidase (2.5 mg), and the chromogen *o*-tolidene (2.5 mg) in 0.01 *M* phosphate buffer (50 ml; p*H* 7.0). Two milliliters of this solution were added to the substrate (*ca.* 50 mg in 2 ml of water), the reaction mixture was incubated at 37 °C and absorbance was monitored at 420 nm with a Gilford automatic recording spectrophotometer. In some experiments the absorbance at a given time was checked after stopping the reaction by addition of glycine buffer (6 ml; 0.25 *M*, p*H* 9.7).

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[9]In this reaction the proportion of 6-*O*-methylthiomethyl ether was much higher (almost 1/4) than in the oxidation of the undeuterated carbinol.

[10]Examination of the reaction mixture prior to acetylation showed that most of this product is formed during the acetylation procedure. The minor product having R_f 0.75 (above) is, in fact, the corresponding, unacetylated, α,β-unsaturated aldehyde (unpublished results).

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