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DIMERIC NATURE OF THE ALDEHYDE PRODUCED FROM METHYL β-D-GALACTOPYRANOSIDE BY D-GALACTOSE OXIDASE

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ABSTRACT

Methyl β-D-*galacto*-hexodialdo-1,5-pyranoside (**2**), produced by the action of D-galactose oxidase on methyl β-D-galactopyranoside, has been characterized in a dimeric form. Structural examination of the peracetate of this dimer by p.m.r. spectroscopy and by analysis of its mass-spectral fragmentation-patterns showed that **2** behaves as a β-hydroxy aldehyde, engaging in unsymmetrical dimerization *via* the 4 and 6 positions; this involves creation of a 1,3-dioxane ring as a bridge between the two units of the dimer. Aldehyde **2** also undergoes ready α,β-elimination.

INTRODUCTION

It had originally been assumed[1] that the action of D-galactose oxidase (1.1.3.9) on D-galactose involves oxidation at the anomeric center to yield D-galactonolactone. However, Horecker and coworkers[2,3] later demonstrated that this enzyme catalyzes aerobic oxidation of the primary carbon atom of the sugar to give a dialdehyde, namely, *galacto*-hexodialdose, and hydrogen peroxide. A variety of D-galactopyranose derivatives are similarly oxidized[2–4]. In recent studies, we have shown that the enzyme specifically abstracts the pro-*S* 6-hydrogen atom of the substrate[5,6], and that the importance of the 4-hydroxyl group of D-galactose is more likely to be a function of its size than of its hydrogen-bonding ability[7]. Related to those studies has been an examination of the oxidation products, the results of which form the subject of this article.

RESULTS AND DISCUSSION

The reaction conditions used for the preparation of products were similar to those for the kinetic experiments described earlier[5], but on a larger scale; in addition, the D-galactose oxidase was coupled with catalase, to promote decomposition of the (enzyme-inhibiting) hydrogen peroxide formed and thereby to favor the oxidation step[2].

At a concentration of 50mM, methyl β-D-galactopyranoside (**1**) was oxidized almost to completion in three hours. According to the results of p.m.r. spectroscopy,

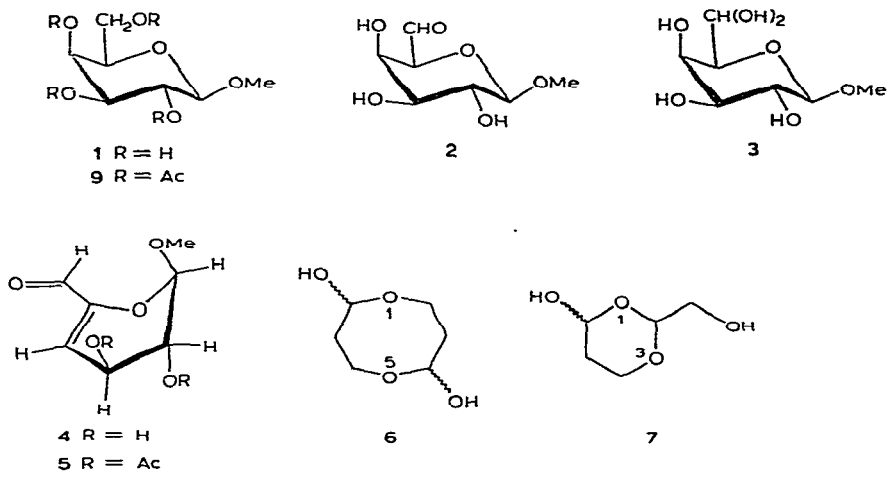
the main product of the reaction appeared to be the expected methyl β-D-*galacto*-hexodialdo-1,5-pyranoside (**2**). Thus, in deuterium oxide solution, a prominent doublet (J 7.5 Hz) at 5.2 p.p.m. could be attributable[8–10] to the hydrated form **3**; in methyl sulfoxide solution, a substantial proportion of the free aldehyde form was present, as shown by a singlet at 9.5 p.p.m. The occurrence of a weak signal at 9.2 p.p.m. and an apparently related one at 6.0 p.p.m. suggested[11,12] the presence of a small proportion of an α,β-unsaturated aldehyde (probably **4**). Further evidence for the latter was provided by thin-layer chromatography (t.l.c.); this showed the presence of a fast-moving (R_F 0.75), minor component that stained deeply with (2,4-dinitrophenyl)hydrazine and absorbed in the ultraviolet (u.v.) region. Enough of this component was isolated by column chromatography to permit determining that its λ_{max} in water is at 250 nm, which is consistent[11] with structure **4**. The major product (**2** or **3**, or both) was detected as a material (R_F 0.63) also staining readily with (2,4-dinitrophenyl)hydrazine, and a third, relatively prominent component (R_F 0.46) also appeared. Undoubtedly, the latter was attributable to dimerization of **2**, as will be discussed.

It appears more likely that **4** is formed by chemical α,β-elimination from the β-hydroxy aldehyde **2**, than by an enzymically catalyzed process. However, "eliminases" are well known among polysaccharide-degrading enzymes[13,14], and the microbial conversion of D-glucose into 6-deoxy-D-glucose possibly involves the intermediacy of an α,β-unsaturated ketone[15,16]. Attempts to establish, more definitively, the origin of **4** were not successful. In any event, it is clear that **2** can undergo facile elimination under other conditions: low-temperature acetylation, with acetic anhydride–pyridine, of the product from the D-galactose oxidase reaction gave a 25% yield of methyl 2,3-di-*O*-acetyl-4-deoxy-6-*aldehydo*-α-L-*threo*-hex-4-eno-1,5-pyranoside[11] (**5**), characterized as its (2,4-dinitrophenyl)hydrazone.

The acetylation just described did not produce a detectable amount of the peracetate of **2** (or **3**) but, instead, a *single* dimeric form (m.p. 206–208°) of the aldehyde hexaacetate (**8**) in 70% yield.

Structure of the dimer acetate. — If compound **2** (or **3**) is considered as the starting point, several modes of dimerization are, in principle, possible, because each aldehyde group may engage in hemiacetal formation with either the 2-, 3-, or 4-hydroxyl group prior to acetylation. Inspection of molecular models indicated, however, that any such combination of two molecules of **2** involving either OH-2 or OH-3 leads to impossible crowding, and that OH-4 is the only junction feasible for inter-unit bonding with an aldehyde group. If, then, compound **2** is regarded as a β-hydroxy aldehyde, there is the possibility that dimerization results either in the formation of a 1,5-dioxocane derivative[17] (**6**), or a 1,3-dioxane[18] (**7**).

The p.m.r. spectrum of the dimer acetate contains two methoxyl methyl signals and a total of six acetate methyl signals and, at 220 MHz, two fully resolved groups of signals attributable to protons 1 to 6 (see Fig. 1). This shows that the dimer has been formed in an unsymmetrical fashion, although, from the spin–spin coupling-pattern, it is clear that each residue has retained a stereochemistry characteristic of a β-D-



galactopyranoside, namely, spacings of about 10 Hz for interactions between protons 1, 2, and 3, and narrower spacings (1–3.5 Hz) for coupling between protons 3, 4, and 5 (see Fig. 1). Because of this bimolecular dissymmetry, a structure analogous to that depicted in formula **6** appears improbable. Except in the unlikely circumstance that the configurations at the two hemiacetal centers are different, the 1,5-dioxo compound would be symmetrical; even if the configurations did differ, such a large difference in

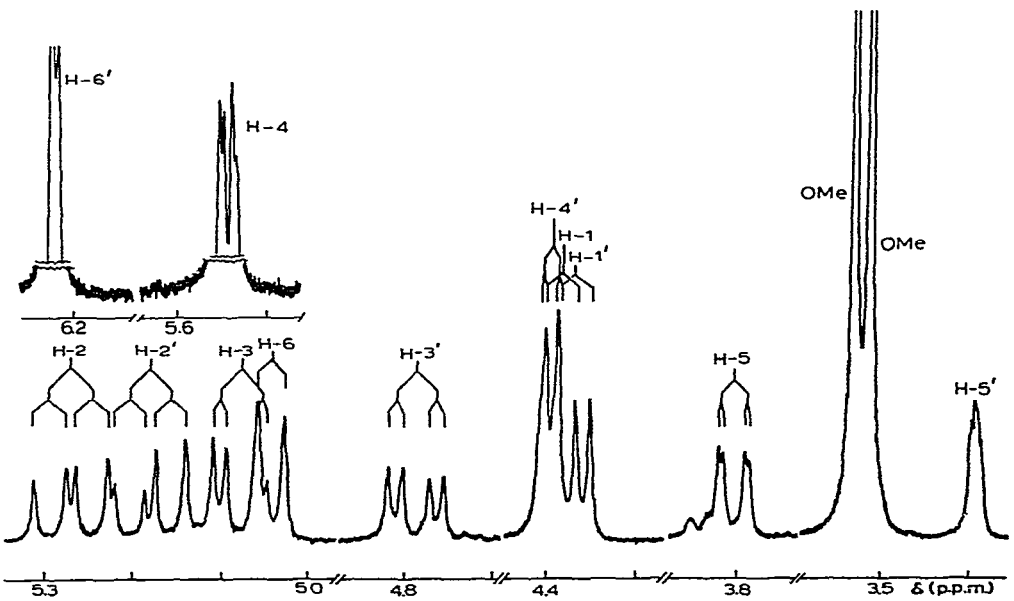
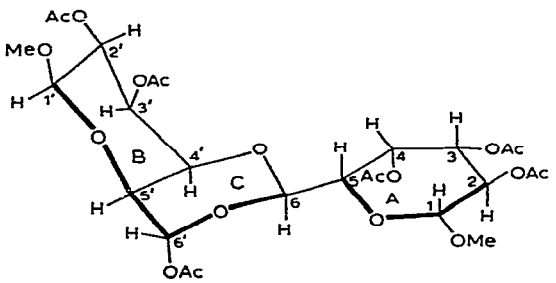


Fig. 1. Partial p.m.r. spectrum (220 MHz, 500-Hz sweep-width) of dimer **8** in $CDCl_3$. Signal H-6' and H-4 insets were recorded at higher gain than the rest of the spectrum.

chemical shift as that observed between corresponding protons of the two residues would hardly be expected. By contrast, when incorporated into the 1,3-dioxane type of structure, two residues of **2** must become distinctly nonequivalent. A fuller examination of the p.m.r. data, and also of the mass-spectral information, showed them to be consistent with the latter possibility and with formulation of the dimer as shown in **8**.



8

Structure **8** requires that ring A be independent of the 1,3-dioxane ring (C). Accordingly, the chemical shifts for protons 1 to 5 are almost the same as those of methyl β-D-galactopyranoside tetraacetate (**9**) (see Table I). It may also be seen that

TABLE I

CHEMICAL SHIFTS (δ) OF RING PROTONS OF DIMER **8** AND METHYL 2,3,4,6-TETRA-*O*-ACETYL-β-D-GALACTOPYRANOSIDE (**9**)[a]

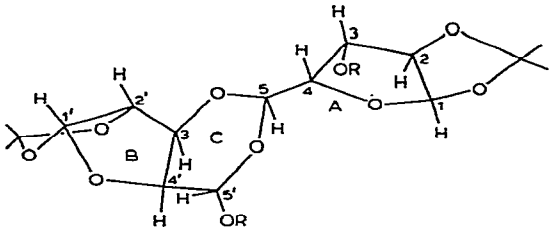
Proton	*For* **9**	*For* **8**	
		Ring A	*Ring B*[b]
H-1	4.40	4.37	4.39
H-2	5.21	5.27	5.18
H-3	5.00	5.08	4.79
H-4	5.49	5.55	4.40
H-5	3.90	3.81	3.39

[a]In $CDCl_3$. [b]H-1′ to H-5′.

chemical shifts of the protons of ring B differ from those of **7**, except for H-1′ and H-2′, which, as depicted, are remote from the junction of rings B and C. In particular, H-4′ resonates more than 1 p.p.m. upfield from H-4 of **7**, consistent both with the absence of an adjacent (4′) acetoxyl group and the participation of O-4′ in the inter-unit linkage; these marked effects are evident to a lesser degree as an increased shielding of H-3′ and H-5′.

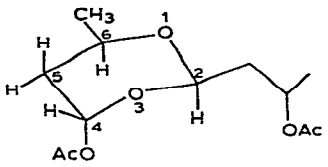
An important feature of the spectrum that is consistent with structure **8** is the appearance of only one 6-proton signal (H-6′, at 6.22 p.p.m.) in the downfield region,

expected for a hemiacetal proton α to an acetoxyl group*. In examining an acetylated dimer of 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,5-furanose (**10**), Schaffer and Isbell[19] noted that the two ester groups are saponified at markedly different rates. This behavior and p.m.r.-spectral data[20] are consistent with structure **10**: the



10 R = Ac

anomeric-like 5′-*O*-acetyl group should be more reactive[19] than the acetyl group at O-3. Although isomeric forms of **10** are possible, it appears, as pointed out by Inch[20], that steric interactions disfavor these other forms. Dimer **8**, it will noted, is closely analogous to compound **10**, and, presumably, its formation is governed by similar factors** (see later). Two isomeric dimers (**11** and **12**, derived acetates) are obtained



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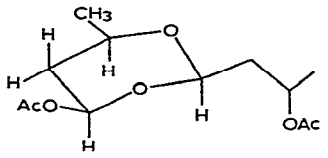

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from aldol, both consistent with a 1,3-dioxane ring-structure[21]. The chemical shift of the anomeric proton of **12** is 5.75 p.p.m., whereas that of **11** is 6.15 p.p.m. As the latter value is close to that (6.22 p.p.m.) for H-6′ of **8**, it supports the assignment of an axial orientation to the 6′-acetoxyl group of **8**; this also applies to H-5′ of **10** which

*The identity of this signal (and that of H-6) was readily confirmed by the spectrum of the dimer acetate obtained when methyl β-D-galactopyranoside-*6-d* (predominantly *6R*) was used as the enzyme substrate[5].

Examination of the mother liquor of **8 failed to reveal an isomeric form of this dimer.

resonates[20] at 6.36 p.p.m. In addition, the small H-5',H-6' coupling (1.3 Hz) observed for **8** supports a gauche disposition for these protons; that is, in 1,3-dioxanes[22–25], the *e,e* arrangement gives rise to couplings of 0.6–1.9 Hz, and the *a,e* arrangement, to couplings of 2.6–3.3 Hz, and an axial 6'-acetoxyl group is also favored by the anomeric effect[26,27].

A stepwise mechanism[28] may be envisaged for the formation of **8**. Inspection of Dreiding models suggested that the addition of OH-4 (of residue B) can take place readily at the *si*-face of the C-6 carbonyl group of residue A. The hydroxyl group of

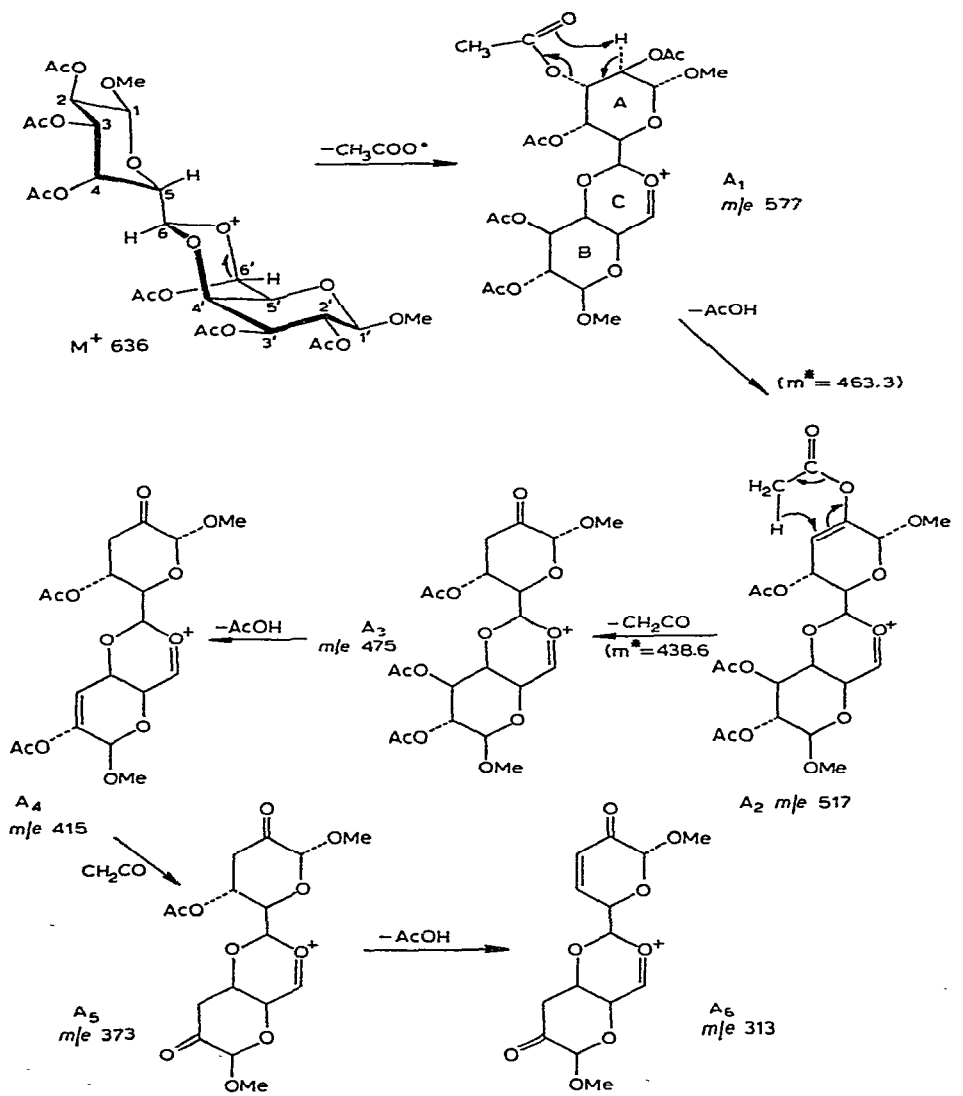

Fig. 2. Mass-spectral fragmentation of dimer **8**. Sequence postulated for A group of fragments.

the intermediate hemiacetal can then add, again from the *si*-face, to the carbonyl group of B, thus smoothly elaborating the 1,3-dioxane chair as depicted in ring C of **8**.

The mass spectrum of compound **8**. — Several fragmentation-patterns were recognized in the mass spectrum of the dimer acetate, and these are in close accord with structure **8**. As examples, Figs. 2 and 3 illustrate schemes that account for two fragmentation sequences, arbitrarily designated A and B; these, as well as the other

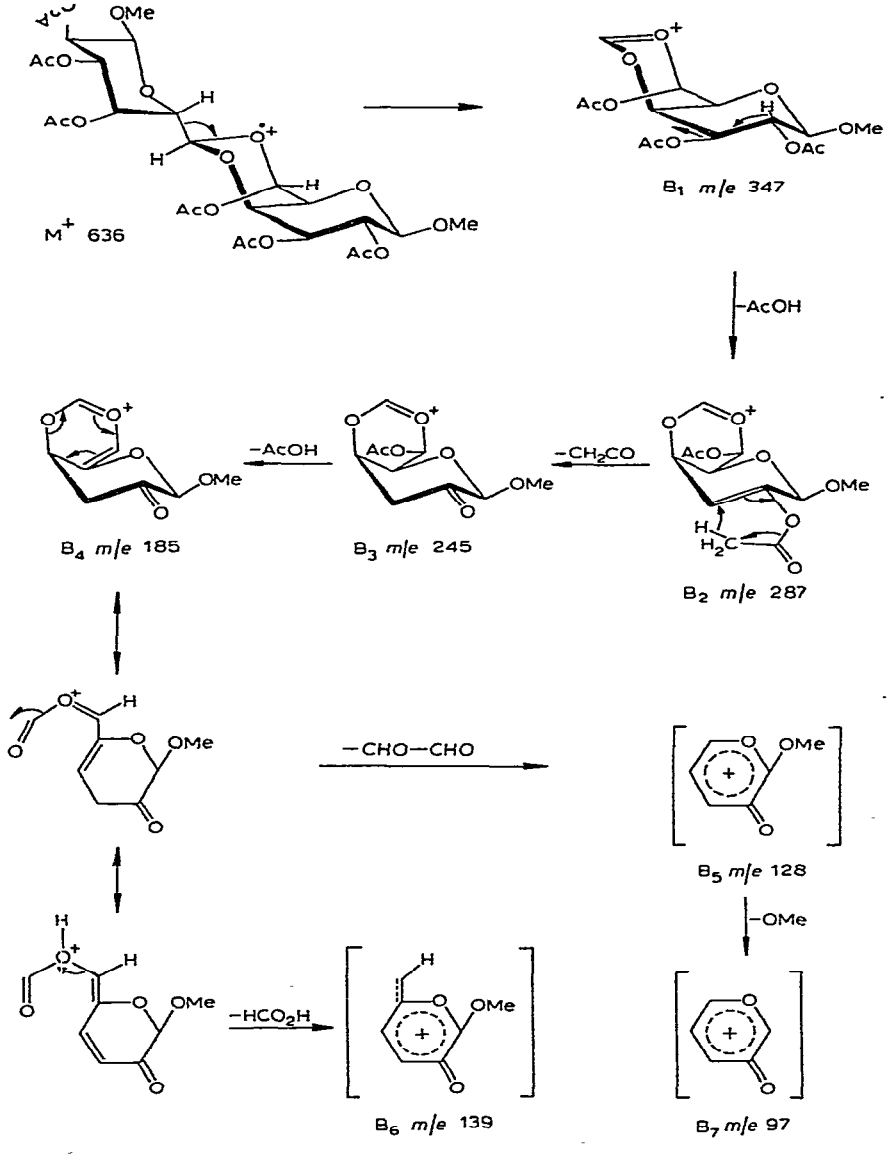

Fig. 3. Mass-spectral fragmentation of dimer **8**. Sequence postulated for B group of fragments.

sequences, show a number of features characteristic of per-*O*-acetylated hexopyranosides[29] and hexopyranoses[30].

An intense peak (A_1; Fig. 2) appears at M-59 mass units, corresponding to loss of an acetoxyl radical (CH_3COO). Probably, the latter is derived from the anomeric, 6′-acetoxyl group, because glycosidic acetate groups are commonly lost as radicals rather than as acetic acid[29]. Loss of acetic acid from the A_1 ion, depicted here as occurring from ring A, yields ion A_2; this transition gives rise to an intense, metastable peak at 463.3 mass units (calculated, 463.3). The conversion of A_1 into A_2 generates a double bond next to an *O*-acetyl group, which is favorable to loss of the latter as ketene[29]. A similar sequence may be depicted, as shown, for ring B_1. Noteworthy in this A series is the fact that, in the mass spectrum of **8** bearing deuterium at C-6 and C-6′ (see footnote, *), the lines are all shifted upward by two mass units.

The B series of ions (see Fig. 3) provides an example of a more extensive fragmentation-sequence. The initial step appears to involve cleavage of the C-6–C-5 bond to give B_1, an ion of strong intensity at *m/e* 347 (or at *m/e* 349 in the spectrum of dideuteriated dimer, which indicates that there is no H–D scrambling in this process). Loss of acetic acid then occurs (B_2), followed by ketene, to yield B_3. Removal of the glycosidic acetate as acetic acid accounts for the formation of B_4. Ions B_6 and B_7 possibly arise from rearrangement of B_4 and subsequent fragmentation. An ion analogous to B_4 (*m/e* 187) is found in the spectrum of the deuteriated dimer, but there is also a strong peak at 186 mass units (which is absent from the spectrum of **10**), suggesting that H–D scrambling occurs at this stage.

EXPERIMENTAL

General. — T.l.c. plates were prepared with Silica Gel G; chromatograms were developed with (*A*) 9:1 (v/v) benzene–ether, or (*B*) 3:2:1 (v/v) propyl alcohol–ethyl acetate–water as the solvent, and visibilized with 5% conc. sulfuric acid in ethanol[31]. Column chromatography was performed with Whatman cellulose powder, or with silica gel (Macherey, Nagel and Co.).

P.m.r. spectra were recorded with a Varian HA-100 spectrometer; spectra at 220 MHz were provided by the Canadian 220-MHz NMR Centre, Sheridan Park, Ontario. Mass spectra were recorded with a double-focusing MS-902 AEI spectrometer operating at 70 eV, with an ion-source temperature varied from 100–200°, a trap current of 500 μA, and an acceleration voltage of 8,000. Electronic spectra were recorded with a Unicam SP-800 ultraviolet spectrometer. Microanalyses were performed by Alfred Bernhardt, Elbach über Engelskirchen, West Germany. Galactose oxidase and catalase were purchased from Worthington Biochemical Corporation.

*Oxidation of methyl β-*D*-galactopyranoside with* D*-galactose oxidase.* — In a typical experiment, the substrate (40 mg) was dissolved in phosphate buffer (4 ml; 0.2M, pH 7.0) and incubated with D-galactose oxidase (5 mg, 125 units) and catalase (5 mg) for 3 h at 37°. T.l.c. examination (solvent *B*) showed the presence of a major

product (R_F 0.63) and two minor products (R_F 0.75 and 0.46, respectively). A mixture of Amberlite IR-120 (H^+) and Dowex-1 (HCO_3^-) resins was added to the digest with stirring, the suspension was filtered, and the filtrate was concentrated and then lyophilized, affording a colorless residue (yield, 36 mg).

Preliminary examination of the oxidation products. — Components of the oxidation product were separated by preparative t.l.c. (solvent *B*), and acetylated (without isolating them from the silica gel). As indicated by the t.l.c. examination and the results of p.m.r. spectroscopy, the zone containing the u.v.-absorbing component (**4**) yielded the α,β-unsaturated aldehyde **5**; the fraction corresponding to aldehyde **2** yielded a mixture of **5** and the dimer **8**; the slowest-moving component yielded mainly **8**, and a small proportion of **5**.

In a separate experiment, the oxidation products were separated by column chromatography on cellulose with 4:1:5 butyl alcohol–ethanol–water (upper layer). The fastest-moving component, corresponding to **4**, showed $\lambda_{max}^{H_2O}$ 253 nm.

*Methyl 2,3-*di-O*-acetyl-4-deoxy-6-*aldehydo-α-L-threo-*hex-4-eno-1,5-pyranoside* (**5**). — The total product of enzymic oxidation, just described, was treated with pyridine (0.8 ml) and acetic anhydride (0.4 ml) for 18 h at room temperature, the mixture was evaporated *in vacuo*, and the syrupy residue was chromatographed on a column of silica gel with solvent *A*. The title compound was eluted first (yield, 10 mg); it was dissolved in ether (0.5 ml) and to this solution were added 3–4 drops of 0.1M (2,4-dinitrophenyl)hydrazine in phosphoric acid–ethanol. Orange–yellow crystals formed rapidly, and were filtered off and washed with ethanol–ether; m.p. 148–152°, undepressed on admixture with a specimen prepared from an authentic sample of the α,β-unsaturated aldehyde[11]. The p.m.r. and mass spectra of the two samples of hydrazone were indistinguishable.

Hexaacetate (**8**) *of dimeric methyl* β-D-galacto-*hexodialdo-1,5-pyranoside.* — Continued irrigation of the column of silica gel afforded methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (3 mg). The eluant was then changed to 1:4 benzene–ether, which effected elution of dimeric compound **8** (30 mg); m.p. (after recrystallization from ethanol) 206–208°, $[\alpha]_D$ +41.6° (*c* 2.1, chloroform).

Anal. Calc. for $C_{26}H_{36}O_{18}$: C, 49.1; H, 5.7; mol. wt. 636. Found: C, 48.6; H, 5.7; M^+ $(M-59=577)^{29,30}$, 636.

The hexa-*O*-acetyl derivative of the dimer, obtained by oxidation of the 6-deuterio analog of **1**, had m.p. 210–211°, $[\alpha]_D$ +47° (*c* 0.4, chloroform).

Anal. Calc. for $C_{26}H_{34}{}^2H_2O_{18}$. C, 48.9; H+^{2}H, 6.0. Found: C, 49.0; H+^{2}H, 5.8.

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