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SYNTHESIS OF 4-DEOXY-D-*xylo*-HEXOSE AND 4-AZIDO-4-DEOXY-D-GLUCOSE AND THEIR EFFECTS ON LACTOSE SYNTHASE

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ABSTRACT

Syntheses are reported of 4-deoxy-D-*xylo*-hexose and 4-azido-4-deoxy-D-glucose as potential inhibitors for lactose synthase [uridine 5′-(α-D-galactopyranosyl pyrophosphate):D-glucose 4-β-D-galactopyranosyltransferase, EC 2.4.1.22]. These syntheses involved SN2 displacement of the 4-methylsulfonyloxy group of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-methylsulfonyl-α-D-galactopyranoside by iodide and azide ions. In both cases, inversion in configuration was observed. The resulting intermediates, methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-iodo-α-D-glucopyranoside and methyl 4-azido-2,3,6-tri-*O*-benzoyl-4-deoxy-α-D-glucopyranoside, were obtained in crystalline form. Both 4-deoxy-D-*xylo*-hexose and 4-azido-4-deoxy-D-glucose were found to be inhibitors for lactose synthase in the presence of α-lactalbumin, but had no effect in the absence of α-lactalbumin. Both D-glucose analogues bind to the enzyme system far more weakly than D-glucose, suggesting that the recognition of the 4-OH group of the acceptor substrate is an important factor in binding.

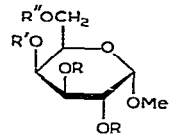
INTRODUCTION

Deoxy sugars and substituted sugars are of particular interest in biochemistry as tools for examining the recognition of sugars by enzymes. In general, oligosaccharides are formed biologically by the transfer of a glycosyl residue from an activated derivative (*e.g.*, a UDP derivative) to a particular group on an acceptor sugar. The position of transfer is, in a number of cases, the 4-position of a monosaccharide. Our interest in the mechanism of lactose synthase [uridine 5′-(α-D-galactopyranosyl pyrophosphate):D-glucose 4-β-D-galactopyranosyltransferase, EC 2.4.1.22], which catalyzes reaction (*1*), has prompted us to investigate the effects of 4-deoxy-D-*xylo*-hexose (**12**) and 4-azido-4-deoxy-D-glucose (**11**) on the steady-state kinetics of this enzyme system.

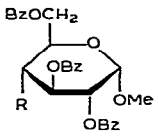
UDP-galactose + D-glucose → lactose + UDP (*1*)

The general properties of lactose synthase have been the subject of recent reviews[1,2]. The enzyme system consists of two components: a catalytic component, galactosyltransferase, and the regulatory protein, α-lactalbumin. In isolation, galacto-

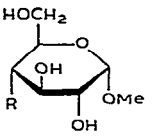
syltransferase catalyzes the transfer of D-galactose from UDP-galactose to OH-4 of a 2-acetamido-2-deoxy-D-glucopyranosyl group in oligosaccharides, glycoproteins, as well as to the free monosaccharide, but it catalyzes lactose synthesis very poorly because of its low affinity for D-glucose (K_m of ~2M). In the presence of α-lactalbumin, the K_m for D-glucose is decreased by three orders of magnitude, so that lactose synthesis is catalyzed effectively with D-glucose concentrations in the physiological range.



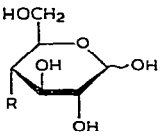
1 R = R′ = R″ = H
2 R = R″ = Bz, R′ = H
3 R = R″ = Bz, R′ = CH_3SO_2
4 R = R″ = Bz, R′ = (*p*)$BrC_6H_4SO_2$
5 R = R″ = Bz, R′ = Ts



6 R = I
7 R = N_3
8 R = H



9 R = N_3
10 R = H



11 R = N_3
12 R = H

As the catalytic action of lactose synthase requires two substrates (UDP-galactose and D-glucose) and two effectors (Mn^{2+} ion and α-lactalbumin), the number of possible steady-state mechanisms is very large. There is general agreement from various studies that the mechanism is a sequential one. Although earlier studies suggested that the mechanism is an ordered one, where the binding of Mn^{2+} and UDP-galactose precedes that of acceptor substrate (D-glucose), different conclusions were drawn as to the nature of enzyme–substrate complexes with which α-lactalbumin associates during the catalytic cycle. On the basis of binding and kinetic studies, Powell and Brew[3] proposed that ordered binding of Mn^{2+} and UDP-galactose is followed by a random, but highly synergistic binding of α-lactalbumin and D-glucose. In contrast to all previous work, Bell *et al.*[4] concluded that both substrates and α-lactalbumin attach to an enzyme–Mn^{2+} complex in a completely random, equilibrium manner.

As the lactose synthase reaction appears to be irreversible, studies of the reverse reaction have not been possible, and the use of products as inhibitors has been of little value. However, the different proposals can be evaluated by the use of substrate-analogue, dead-end inhibitors; UDP-glucose, a competitive inhibitor with respect to UDP-galactose, has been extensively used for this purpose, but a satisfactory acceptor-substrate analogue that is a competitive inhibitor has not hitherto been available, despite the examination of a number of monosaccharides.

Therefore, we decided to synthesize and examine the effects of two D-glucose derivatives modified at C-4, **11** and **12**, in an attempt to resolve some of the controversy surrounding the mechanism of lactose synthase. Several syntheses of 4-deoxy-D-*xylo*-hexose (**12**, 4-deoxy-D-glucose) and its derivatives have been reported[5–8]. The most commonly used route for the synthesis of **12** is based on the cleavage of the epoxide ring of methyl 3,4-anhydro-α-D-galactopyranoside with hydrogen in the

presence of Raney nickel, and the separation of the resulting 3- and 4-deoxy derivatives which are subsequently hydrolyzed to free sugars[9]. We decided to follow a more direct route, suggested by Hanessian[10] which, apart from giving other intermediates of practical usefulness for our study, also avoided the possibility of contaminating the final product with 3-deoxy-D-*ribo*-hexose (3-deoxy-D-glucose). The approach involves the SN2 displacement, with iodide ion, of a 4-sulfonyloxy group of a suitably protected derivative of methyl α-D-galactopyranoside, and its hydrogenation to yield the deoxy derivative. Although a report of obtaining **11** in low yield by use of 1,2,3,6-tetra-*O*-acetyl-4-*O*-mesyl-α-D-glucose as the starting material has been published[11], the reaction conditions for obtaining the iodo derivative (with sodium iodide in acetone at 138° for 48 h in a sealed steel tube) were inconvenient and drastic, and no attempt was made to characterize the intermediate iodo derivative. Similarly, **11** was obtained by displacement at C-4 with azide ion. Successful displacement at the 4-position with the azide ion has been previously reported[12,13], but attempts to obtain the reducing form of the azido derivative were unsuccessful[12]. In general, the synthesis closely resembles those of related derivatives in other series[13] and, therefore, we restricted ourselves in the discussion to those results and observations that are particularly characteristic of the present work.

RESULTS AND DISCUSSION

Partial benzoylation of methyl α-D-galactopyranoside (**1**) afforded mainly the 2,3,6-tribenzoate derivative[12] (**2**). Treatment of **2** with methanesulfonyl chloride, *p*-bromobenzenesulfonyl chloride, and *p*-toluenesulfonyl chloride gave **3**, **4**, and **5**, respectively, as the starting compounds for the SN2 reactions. Unlike methyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside, where the *p*-bromophenylsulfonyloxy derivative was reported to be most useful for carrying out an SN2 reaction with iodide ion in acetonitrile[13], **4** under the same conditions did not undergo significant conversion even after 48 h. However, **3** was found to undergo selective displacement, in high yield, at C-4 by sodium iodide with inversion by performing the reaction in *N*,*N*-dimethylformamide under a nitrogen atmosphere at 120°, to give mainly **6**. Since **4** and **5** were not tested under the same conditions, no specific conclusion could be drawn about the reactivity of different leaving groups. It is interesting to note that, in case of the corresponding D-*gluco* derivative, the iodide displacement was accompanied by appreciable halogen-exchange resulting in overall retention of configuration[13-15]. It appears that an iodo substituent at C-4 is more stable in the D-*gluco* than in the D-*galacto* configuration.

Similarly, treatment of **3** with sodium azide in *N*,*N*-dimethylformamide at 110° gave methyl 4-azido-2,3,6-tri-*O*-benzoyl-4-deoxy-α-D-glucopyranoside (**7**) in good yield. As observed earlier[12], reaction at higher temperatures gave only an oily product from which crystalline **7** could not be obtained. The n.m.r. study of **6** and **7** clearly indicates that the displacement of the sulfonyloxy group at C-4 with iodide and azide ions, respectively, has taken place with inversion of configuration, the values of

TABLE I

^{1}H-N.M.R. DATA FOR COMPOUNDS **6** AND **7**

	Compounds	
	6	**7**
Chemical shifts (δ)		
H-1	5.25	5.17
H-2	5.19	5.24
H-3	6.16	6.03
H-4	4.31	3.89
H-5	4.49	4.09
H-6*a*	4.90	4.66
H-6*b*	4.80	4.70
CH_3	3.46	3.45
Ar	7.0–8.30	7.20–8.30
Apparent coupling constants (Hz)		
$J_{1,2}$	3.8	3.8
$J_{2,3}$	9.7	9.2
$J_{3,4}$	10.0	9.2
$J_{4,5}$	11.5	10.5
$J_{5,6a}$	2.2	2.8
$J_{5,6b}$	4.0	3.8
$J_{6a,6b}$	12.5	12.3

$J_{3,4}$ and $J_{4,5}$ ~10 Hz indicating the D-*gluco* configuration. For the D-*galacto* configuration, these values are much lower[13].

Compound **6** could be readily hydrogenated to yield the corresponding 4-deoxy derivative **8** in crystalline form. Treatment of **7** and **8** with methanolic sodium methoxide at room temperature afforded the corresponding *O*-debenzoylated derivatives **9** and **10**. Compound **10** was purified by column chromatography with Bio-Gel P-2. Measurements of the optical rotation of the fractions showed a symmetrical, single peak. The free sugars **11** and **12** were obtained by mild, acid hydrolysis of **9** and **10**, respectively, in the presence of ion-exchange resin IR-120 (H^+), followed by purification on a column of Bio-Gel P-2, which gave an excellent separation of the free reducing-sugars from the unhydrolyzed compounds[16]. Whereas 4-deoxy-D-*xylo*-hexose (**12**) could be obtained in crystalline form, 4-azido-4-deoxy-D-glucose (**11**) was found to be hygroscopic and could not be crystallized from a solvent. However, after careful purification with Bio-Gel P-2, it could be dried under vacuum to give a colorless, crystalline solid that gave a satisfactory elemental analysis and was found to be homogeneous on paper in several solvent systems. H.p.l.c. with an analytical carbohydrate column also indicated that **11** and **12** were compounds free from impurities. Both gave single peaks in 83% acetonitrile, **11** at 1.97 and **12** at 3.54 min, while D-glucose and D-galactose were eluted at 5.80 and 5.97 min, respectively.

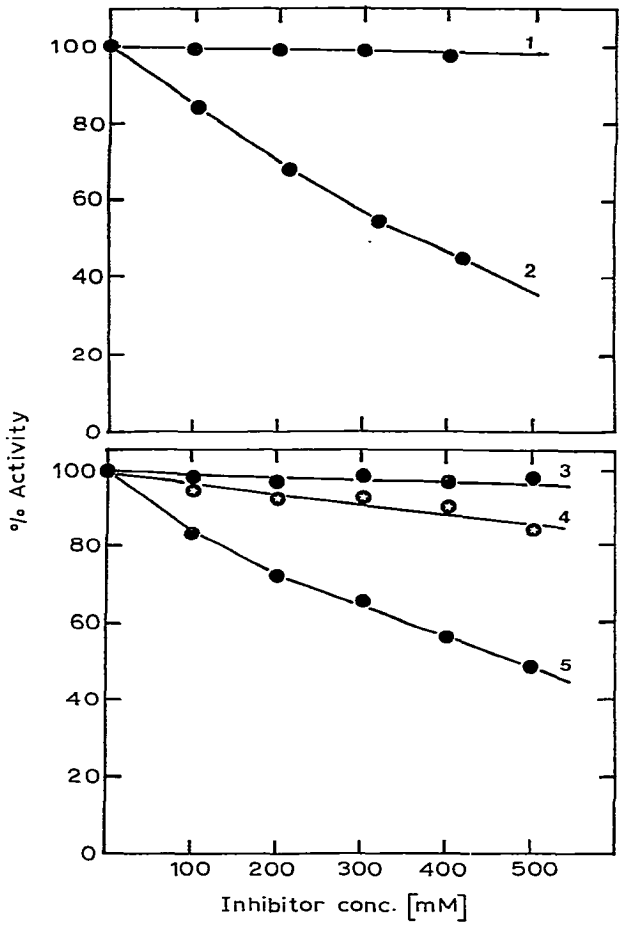


Fig. 1. The effect of the concentration of **11**, **12**, and D-galactose on lactose and *N*-acetyllactosamine synthesis. The velocities were measured at 37° in the presence of 2.0mM Mn^{2+}, 0.1% bovine serum albumin, and 50.0mM cacodylate buffer (pH 7.4). The concentration of 2-acetamido-2-deoxy-D-glucose was 5.0mM for *N*-acetyllactosamine synthesis in the absence of α-lactalbumin, and the concentrations of D-glucose and α-lactalbumin were 2.5mM and 12.5μM, respectively, for lactose synthesis. (1) Effect of **11** on *N*-acetyllactosamine synthesis; (2) effect of **11** on lactose synthesis; (3) effect of **12** on *N*-acetyllactosamine synthesis; (4) effect of D-galactose on lactose synthesis; and (5) effect of **12** on lactose synthesis.

4-Deoxy-D-*xylo*-hexose (**12**) and 4-azido-4-deoxy-D-glucose (**11**), as well as D-galactose, were tested for inhibitory effects on the activity of galactosyltransferase, in the absence of α-lactalbumin with 2-acetamido-2-deoxy-D-glucose as substrate, and in the presence of α-lactalbumin with D-glucose as substrate. At the same time, their possible abilities to act as substrates for the enzyme system were examined. Both derivatives (and D-galactose) were completely inactive as substrates in the presence or absence of α-lactalbumin. In the absence of α-lactalbumin, both **11** and **12** showed no significant inhibitory effects with 2-acetamido-2-deoxy-D-glucose as

substrate, whereas in the presence of α-lactalbumin, both derivatives showed pronounced inhibition of lactose synthesis. D-Galactose was a very poor inhibitor of lactose synthesis (Fig. 1). The concentration dependence of the inhibitory effects of **11** and **12** suggests that both compounds bind to the enzyme far more weakly than D-glucose under the same conditions. Considered together with the extremely poor inhibitory effects of D-galactose, this would suggest that the recognition of the OH-4 group of the acceptor substrate is an important factor in binding to the enzyme. Although more-detailed kinetic studies are required to clarify the effects of these two D-glucose analogs on lactose synthase, it is clear that they may be useful probes for the study of the specificity and of the mechanism of lactose synthase, and possibly of other D-glucose-utilizing enzymes.

EXPERIMENTAL

General. — Galactosyltransferase (mol. wt. 50,000) was purified from bovine colostrum, and α-lactalbumin from bovine milk as described previously[1,2]. In kinetic studies, initial velocities were measured by use of a standard radiochemical method[1,2]. Chromatographic-grade silica gel was used for nonaqueous- and Bio-Gel P-2 (100–200 mesh) for aqueous-phase column chromatography. Thin-layer chromatographic plates were precoated with Silica Gel GF (thickness 250 microns). Paper chromatographs were run by the descending technique on Whatman 3MM chromatographic paper and reducing sugars were detected by the standard silver nitrate method. All melting points reported are uncorrected and were determined with a Fisher–Johns apparatus. Optical rotations were determined with a Rudolph polarimeter, i.r. spectra were recorded with a Perkin–Elmer 721 instrument for KBr pellets, and the purity of both final products was controlled with a Waters Associates HPLC instrument equipped with an analytical carbohydrate column. N.m.r. spectra were recorded with a Varian HA-100D-15 instrument operating at 100 MHz. All solutions were evaporated to dryness under reduced pressure at <40°. Satisfactory elemental analyses were obtained for all compounds previously described in the literature.

*Methyl 2,3,6-tri-*O*-benzoyl-α-*D*-galactopyranoside* (**2**) *and methyl 2,3,6-tri-*O*-benzoyl-4-*O*-methylsulfonyl-α-*D*-galactopyranoside* (**3**). — Both compounds were prepared by the method described by Reist *et al.*[12], with the modification that **2** was further purified by column chromatography on silica gel with 9 : 1 (v/v) benzene–ether as the solvent.

*Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-iodo-α-*D*-glucopyranoside* (**6**). — To a solution of **3** (13.5 g) in analytical grade *N,N*-dimethylformamide (120 mL), dry crystalline sodium iodide (22.0 g) was added, and the mixture was heated at 120° with stirring under a dry nitrogen atmosphere. The reaction was monitored by t.l.c. (9 : 1, v/v, benzene–ether), which indicated the progressive formation of a fast-moving compound. After 18 h, the incomplete reaction was stopped. Prolonged heating or heating at a higher temperature resulted in a number of slow-moving products. The reaction mixture was cooled to room temperature, and the salts precipitated by

adding cold ether (200 mL). The suspension was filtered, and the precipitate washed with another 100 mL of ether. Evaporation of the combined filtrates to dryness under reduced pressure gave a tan-colored solid which contained some free iodine. The solid was redissolved in toluene (~25 mL), and applied to a silica column (3.2 × 70 cm) pre-wetted with toluene, and eluted with 19:1 (v/v) toluene–ether. Fractions (15 mL) were collected. T.l.c. in 19:1 (v/v) benzene–ether showed that fractions 11–31 contained the fast-moving major product in a pure form, and fractions 32–40 a mixture of that product and of starting material. Most of the free iodine was adsorbed on the column. Fractions 11–31 were pooled and dried to give a light-colored solid. Crystallization from ether–petroleum ether afforded white, crystalline **6** (8.1 g, 60%). Recrystallization from 99.9% ethanol gave the analytical sample, m.p. 158°, ν_{max}^{KBr} 1730 (C=O), 1290, 1282 (benzoate C-O-C), a weak band at 763, and none at 848 cm^{-1}, which has been reported[17] to be characteristic of **3**.

Anal. Calc. for $C_{28}H_{25}IO_8$: C, 54.54; H, 4.09; I, 20.59. Found: C, 54.70; H, 4.26; I, 19.71.

*Methyl 4-azido-2,3,6-tri-*O*-benzoyl-4-deoxy-α-*D*-glucopyranoside* (**7**). — To a solution of **3** (10.0 g) in *N,N*-dimethylformamide (100 mL), sodium azide (5.0 g) was added, and the mixture was heated for 6 h with stirring at 110–115°. The reaction mixture was cooled to room temperature, the salt precipitated with ether, and the suspension filtered. The filtrate was dried to a tan-colored syrup, which was redissolved in toluene (20 mL) and applied to a silica column (2.5 × 76.2 cm). Elution with 49:1 (v/v) toluene–ether separated the product from the starting material, as monitored by t.l.c. in 19:1 (v/v) benzene–ether. The fractions containing **7** were pooled and dried to a white, crystalline material. Crystallization from 99.9% ethanol afforded 6.5 g (72.7%) of **7**; recrystallization from 99.9% ethanol gave the analytical sample, m.p. 104–105°, $[\alpha]_D^{20}$ +198.5° (*c* 1.0, chloroform); ν_{max}^{KBr} 2128 (N_3), 1730 (C=O), and 1274 cm^{-1} (benzoate C-O-C).

Anal. Calc. for $C_{28}H_{25}N_3O_8$: C, 63.26; H, 4.74; N, 7.91. Found: C, 63.43; H, 4.94; N, 7.70.

*Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-*D*-*xylo*-hexopyranoside* (**8**). — To a solution of **6** (6.0 g) in 99.9% ethanol (350 mL) was added fresh Raney nickel (~15 g), and the suspension was hydrogenated at 3 atm. for 6 h at room temperature. The solution was filtered to remove the catalyst, concentrated under reduced pressure, and kept at room temperature to give **8** as colorless needles. Recrystallization from 99.9% ethanol gave 4.0 g (82.3%) of **8**, m.p. 105°, $[\alpha]_D^{20}$ +129.9° (*c* 2.5, chloroform); t.l.c. (9:1, v/v, benzene–ether): single product, R_F between those of **6** and **3**; ν_{max}^{KBr} 1730 (C=O), 1290, and 1279 cm^{-1} (benzoate C-O-C).

*Methyl 4-azido-4-deoxy-α-*D*-glucopyranoside* (**9**). — Debenzoylation of **7** was accomplished by treatment with sodium methoxide in methanol for 12 h at room temperature. The product was extracted as described by Reist *et al.*[12] to give a crude fraction, which on crystallization from water (1 part), acetonitrile (4 parts), and ether added to turbidity, afforded 1.9 g (84%) of **9**, m.p. 106° (lit.[12] m.p. 108°), $[\alpha]_D^{20}$ +264.35° (*c* 1.0, methanol); ν_{max}^{KBr} 3413, 2941 (OH), and 2110 cm^{-1} (N_3).

*Methyl 4-deoxy-α-*D*-xylo-hexopyranoside* (**10**). — To a solution of **8** (7.5 g) in dry methanol (50.0 mL) was added sodium methoxide (2.7 g) and the mixture was gently boiled for 2 h under reflux, protected from moisture. Processing of the reaction mixture as described earlier afforded a light-colored syrup. The syrup was redissolved in water (2–3 mL) and applied to a column (2.0 × 115 cm) of Bio-Gel P-2, which was eluted with water, and 5.3-mL fractions were collected. Determination of the optical rotation of the fractions gave an elution profile showing a symmetrical single peak. The fractions were pooled and concentrated under reduced pressure at <35° to give a colorless, thick syrup, from which **10** crystallized slowly, at room temperature (yield, 1.46 g, 58.8%), m.p. 90° (lit.[6] m.p. 89–90°), $[\alpha]_D^{20}$ +167.8° (*c* 1.12, methanol) [lit.[6] $[\alpha]_D$ +167.3° (*c* 1.0, methanol)]; no (C=O) absorbance in the i.r. spectrum.

*4-Azido-4-deoxy-*D*-glucose* (**11**). — A solution (50 mL) of **9** (1.8 g) in water was heated at 90° with Amberlite IR 120 (H^+) ion-exchange resin, and the progress of the reaction monitored by measuring the optical rotation. After 6 h, the reaction was stopped, and the suspension filtered to remove the resin. The filtrate was passed through AGI-X8 (CO_3^{2-}) anion-exchange resin, and then treated with charcoal and concentrated to a thick syrup. The syrup was redissolved in water (3 mL) and applied to a Bio-Gel P-2 column, and 5-mL fractions were collected. The fractions were spotted on paper and assayed for the presence of reducing sugar. Determination of the optical rotation showed a small, well-separated peak of unhydrolyzed material preceding the reducing-sugar peak. The reducing-sugar peak was pooled to give a clear solution, which was dried under reduced pressure at <35° to yield **11** (0.84 g, 50%) as hygroscopic, colorless, crystalline material, $[\alpha]_D^{20}$ +120.15° (*c* 2.0, water, equil.); ν_{max}^{KBr} 3390, 2941 (OH), and 2105 cm^{-1} (N_3); paper chromatography(12:5:4, v/v, ethyl acetate–pyridine–water): single component, R_F 2.26 (3 h).

Anal. Calc. for $C_6H_{11}N_3O_5$: C, 35.13; H, 5.37; N, 20.49. Found: C, 35.08; H, 5.63; N, 20.25.

*4-Deoxy-*D*-xylo-hexose* (**12**). — Hydrolysis of **10** (1.0 g) with Amberlite IR-120 (H^+) ion-exchange resin in aqueous solution for 6 h at 90° resulted in its quantitative conversion to the free sugar. The solution was treated with charcoal, concentrated, and separated by gel filtration with Bio-Gel P-2. The resulting clear solution was concentrated to a thick syrup, which at room temperature slowly crystallized to give 0.52 g (56%) of **12**. The crystals were washed with hot ethyl acetate and dried in vacuum; m.p. 130° (lit.[5] m.p. 131–132°), $[\alpha]_D^{20}$ +60.5° (equilibrium after 4 h, *c* 2.05, water) (lit.[5] +60.3°); single, reducing-sugar spot in paper chromatography (12:5:4, v/v, ethyl acetate–pyridine–water): R_F 0.67 (D-glucose 0.53, D-galactose 0.48); (9:5:7, v/v, butanol–pyridine–water): R_F 0.63 (D-glucose 0.52).

Anal. Calc. for $C_6H_{12}O_5$: C, 43.89; H, 7.37. Found: C, 43.95; H, 7.54.

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