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THE INEFFECTIVENESS OF ANALOGS OF D-GALACTAL AS COMPETITIVE INHIBITORS OF, AND SUBSTRATES FOR, β-D-GALACTOSIDASE FROM *Escherichia coli*

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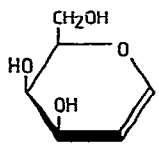
ABSTRACT

2,6-Anhydro-3-deoxy-*aldehydo*-D-*lyxo*-hept-2-enose (**7**) and 2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enitol (**8**) were synthesized as half-chair analogs of D-galactal (**1**). As **1** is a strong inhibitor of, as well as a substrate for, β-D-galactosidase from *Escherichia coli*, the same properties were expected for **7** and **8**; however, both were ineffective. This result, together with those of other authors, allows speculative conclusions on the tight binding of **1** to the enzyme only, when water or an alcohol is bound as a co-substrate.

INTRODUCTION

D-Galactal (**1**) was shown by Lee[1] to be an effective inhibitor of β-D-galactosidase from *E. coli*, a finding that was confirmed, and analyzed in more detail, by Wentworth and Wolfenden[2]. A reasonable explanation for the low K_i value of D-galactal is its resemblance to the potential, half-chair intermediate 1,2-anhydro-D-galactopyranose[3] (**2**).



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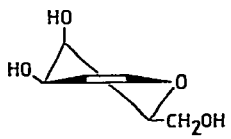

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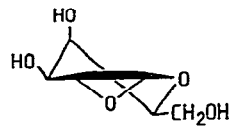


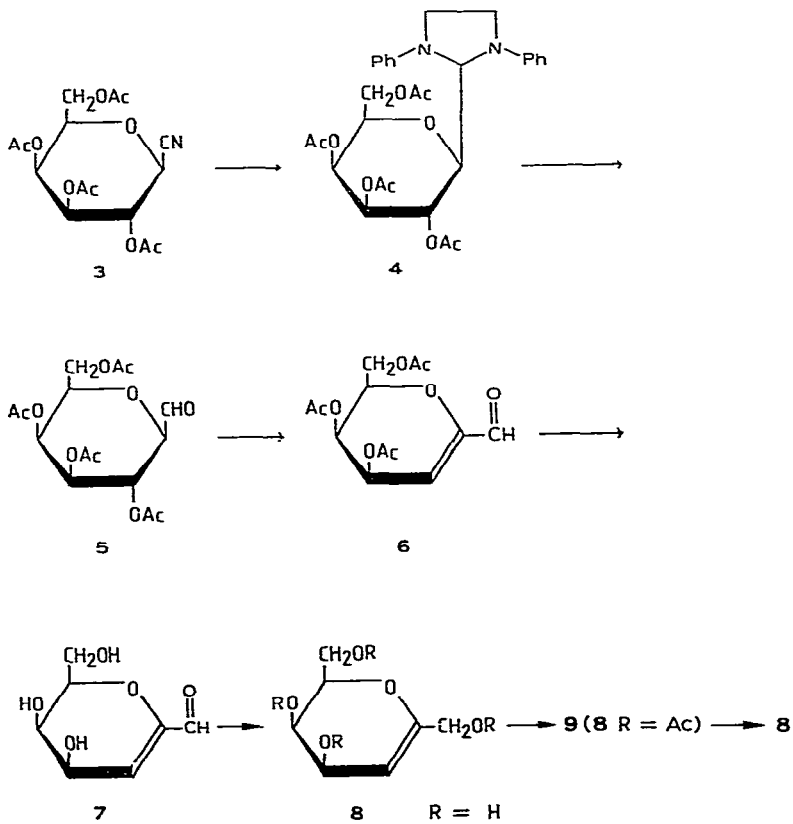
2

Recent investigations have indicated that the intermediate formation of **2** in the hydrolysis of β-D-galactosides by β-D-galactosidase is very likely[4]. Were only a half-chair conformation of D-galactal responsible for its strong inhibitory effect, substitution at C-1 should not alter this property extensively. In order to elucidate the potential influence of groups other than hydrogen attached to C-1 of D-galactal, the analogs 2,6-anhydro-3-deoxy-*aldehydo*-D-*lyxo*-hept-2-enose (**7**) and 2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enitol (**8**) were synthesized, and their interaction with β-D-galactosidase was studied.

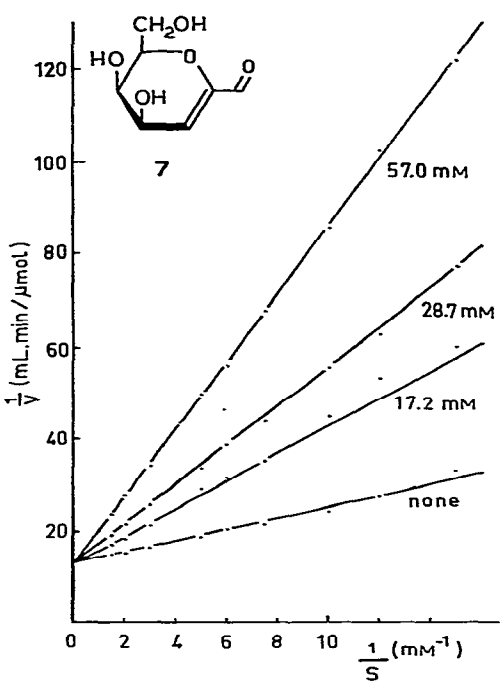
RESULTS AND DISCUSSION

Starting from 2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl cyanide (**3**)[5], 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-*aldehydo*-D-*glycero*-L-*manno*-heptose (**5**) was prepared *via* 1,3-diphenyl-2-(tetra-*O*-acetyl-β-D-galactopyranosyl)imidazolidine (**4**) according to a method of Moffatt and co-workers[6]. On standing in pyridine–acetic acid, compound **5** eliminates acetic acid, to give 4,5,7-tri-*O*-acetyl-2,6-anhydro-3-deoxy-*aldehydo*-D-*lyxo*-hept-2-enose (**6**). By deacetylation of **6**, **7** was obtained; on reduction with sodium borohydride, **7** yielded **8**, which was purified by way of its distillable tetra-acetate (**9**).

An interesting consequence of the interaction of D-galactal with the active site of β-D-galactosidase is its enzyme-catalyzed conversion into 2-deoxy-D-*lyxo*-hexopyranose and 1-deoxyglycerol-1-yl 2-deoxy-β-D-*lyxo*-hexopyranoside, respectively, by *trans*-addition of water, or glycerol, to the enolic double bond[7,8]. Compound **7**



remained unchanged on incubation with β-D-galactosidase for up to 7 days. In phosphate buffer, pH 6.8, compound **8** showed slow, spontaneous hydration which could not, however, be accelerated by β-D-galactosidase. This result is not surprising in the case of **7**, where the carbon–carbon double bond in conjugation with a carbonyl



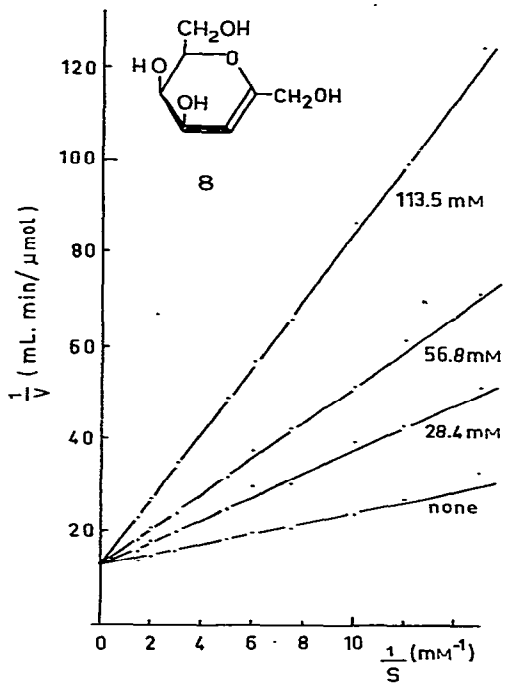


Fig. 1 (left). Determination of the inhibition constant K_i for 2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enose (**7**) in 0.05M sodium phosphate buffer (pH 6.8) containing mM $MgCl_2$, at 30°. The concentrations of substrate *o*-nitrophenyl β-D-galactopyranoside and inhibitor were varied as indicated. The reaction was started by adding ~0.08 U of β-D-galactosidase per mL.

Fig. 2 (right). Determination of the inhibition constant K_i for 2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enitol (**8**). For conditions, see legend to Fig. 1.

group might resist protonation, *e.g.*, electrophilic addition by the enzyme. The behavior of compound **8**, however, should not differ much from that of D-galactal (**1**); here, protonation should occur with even greater ease, as a tertiary carboxonium ion would be formed. Similarly surprising is the fact that **7** and **8**, although competitive inhibitors (see Figs. 1 and 2), show inhibition constants about three orders of magnitude larger than that found for D-galactal (see Table I). Lack of reactivity, at least in the case of **8** could, therefore, be explained by very poor binding to the active site.

It is difficult to find an explanation for the weak binding of the two analogs of D-galactal. Steric reasons can probably be excluded, as 1-thio-D-galactosides having an aglycon of comparable bulk are very good inhibitors[9]. Lack of polarity, however, could well be the cause. The lag phase for the tight binding of D-galactal found by Wentworth and Wolfenden[2] might be due to initial, weak binding to the enzyme (E), with constants comparable to those found for **7** and **8**. Tight binding occurs only when a molecule of water or a suitable alcohol is bound as a co-substrate (see complexes 1 and 2). The binding of a co-substrate would be prevented when a hydroxymethyl (compound **8**) or carbonyl group (compound **7**) blocks its binding

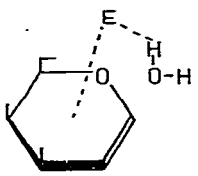
TABLE I

INHIBITION CONSTANTS FOR COMPOUNDS **1**, **7**, AND **8**

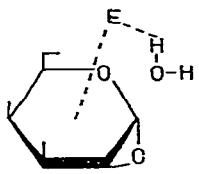
Compound	*Inhibition constant (mM)*
D-Galactal (**1**)	0.05[a], 0.014[b]
2,6-Anhydro-3-deoxy-D-*lyxo*-hept-2-enose (**7**)	11
2,6-Anhydro-3-deoxy-D-*lyxo*-hept-2-enitol (**8**)	22

[a]Ref. 1. [b]Ref. 2.

site. The synergistic effect by the co-substrate would agree well with the hypothesis of the D-galactal–1,2-anhydro-D-galactose analogy[3].



complex 1



complex 2

Complex 2 represents the state after the aglycon has been released from the β-D-galactoside, and the hydroxyl group from water has taken the place of the aglycon alcohol residue*. The tight binding of intermediate and co-substrate in complex 2 is biologically sensible, as release of the intermediate is not possible before reaction with the co-substrate has taken place. The sensitivity of the rate of binding of D-galactal to deuterium oxide[2] is also readily explained by assuming the formation of a tight complex involving the enzyme, D-galactal, and water.

EXPERIMENTAL

General methods. — Melting points are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. T.l.c. was performed on silica gel F_{254} (Merck) with 25:14:7 (v/v/v) ethyl acetate–2-propanol–water for compounds having free hydroxyl groups, and 4:1 (v/v) ether–light petroleum (b.p. 60–70°) for fully protected compounds. Detection was effected by charring with sulfuric acid. I.r. and ^{1}H-n.m.r. (internal standard, Me_4Si) data were obtained with a Perkin–Elmer Infracord, Model 137, and Varian A-60 D and Bruker WH 90 spectrometers, respectively.

1,3-Diphenyl-2-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)imidazolidine (**7**). — Raney nickel (70 mL, ~42 g; from an aqueous suspension) was added at room temperature to a vigorously stirred solution of pyridine (160 mL), acetic acid (95 mL),

*In complexes 1 and 2, water can be replaced by an alcohol.

and water (95 mL) and then sodium hypophosphite monohydrate (25 g, 236 mmol), *N,N'*-diphenylethylenediamine (11 g, 82 mmol), and 2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl cyanide (**3**) (10 g, 28 mmol) were added, the reaction temperature not being allowed to rise above 40°. After 2 h, insoluble material was filtered off under suction, and washed with dichloromethane (4 × 300 mL). (The residue, which is pyrophoric and highly reactive, must be deactivated immediately by mixing with water.) The organic layer of the filtrate was separated, washed with water (6 × 200 mL) until the washings were no longer colored green, dried (anhydrous magnesium sulfate), and evaporated under diminished pressure, residual pyridine being removed by repeated co-evaporation with toluene (3 × 50 mL). The final syrup crystallized when methanol was added. Recrystallization from methanol yielded **4** (11.5 g, 72%), m.p. 153°, $[\alpha]_{578}^{22}$ +12° (*c* 1.0, $CHCl_3$); ν_{max}^{KBr} 1740 (C=O), 1590 (Ph), and 1490 cm^{-1} (Ph); ^{1}H-n.m.r. data ($CDCl_3$): δ 1.87, 2.00, and 2.11 (3 s, 12 H, 4 OAc), and 6.7–7.5 (m, 10 H, 2 Ph).

Anal. Calc. for $C_{29}H_{34}N_2O_9$: C, 62.81; H, 6.18; N, 5.05. Found: C, 62.79; H, 6.32; N, 5.33.

3,4,5,7-Tetra-O-*acetyl-2,6-anhydro*-aldehydo-D-glycero-L-manno-*heptose* (**5**). — To a solution of compound **4** (18 g, 32.5 mmol) in dichloromethane (400 mL) was added a solution of *p*-toluenesulfonic acid monohydrate (17 g, 89.5 mmol) in acetone (100 mL), with stirring. After 20 min, the precipitate was filtered off with a filter aid, and the filtrate was diluted with dichloromethane (400 mL), washed with water (3 × 200 mL), dried (anhydrous magnesium sulfate), and evaporated under diminished pressure at room temperature, to yield a syrup that crystallized from cold ether; yield: 4.4 g (37%), m.p. 118.2°, $[\alpha]_{578}^{22}$ + 38.0° (*c* 1.0, $CHCl_3$); ν_{max}^{KBr} 1740 cm^{-1} (C=O); ^{1}H-n.m.r. data ($CDCl_3$): δ 1.9 (d, 9 H, 3 OAc), 2.09 (s, 3 H, 1 OAc), and 9.28 (s, 1 H, CHO).

Anal. Calc. for $C_{15}H_{20}O_{10}$: C, 50.00; H, 5.59. Found: C, 49.89; H, 5.80.

4,5,7-Tri-O-*acetyl-2,6-anhydro-3-deoxy*-aldehydo-D-lyxo-*hept-2-enose* (**6**). — A solution of compound **5** (10 g, 27.8 mmol) in a mixture of pyridine (50 mL) and acetic acid (50 mL) was kept for ~5 days at room temperature; conversion into **6** (t.l.c.) was then complete. The mixture was evaporated to dryness under diminished pressure, traces of pyridine were removed by co-evaporation with toluene, and the resulting solid was twice recrystallized from ether, and then from cyclohexane, to give colorless needles; yield: 6.25 g (75%); m.p. 116.1°, $[\alpha]_{578}^{22}$ —161° (*c* 1.0, $CHCl_3$); ν_{max}^{KBr} 1750 (C=O ester), 1680 (C=O, α,β-unsaturated aldehyde), and 1650 cm^{-1} (C=C in conjugation with aldehyde group); ^{1}H-n.m.r. data ($CDCl_3$): δ 2.13 (d, 9 H, 3 OAc) and 9.22 (s, 1 H, CHO).

Anal. Calc. for $C_{13}H_{16}O_8$: C, 52.00; H, 5.37. Found: C, 52.34; H, 5.73.

2,6-Anhydro-3-deoxy-aldehydo-D-lyxo-*hept-2-enose* (**7**). — Compound **6** (3.0 g, 10 mmol) was deacetylated by dissolution in 0.02M methanolic sodium methoxide solution (20 mL). After 6 h, the mixture was filtered through silica gel (~20 g in a small column), and organic material was completely eluted with methanol. Decolorization with active charcoal, followed by evaporation under diminished pressure

at room temperature, yielded a solid which was twice recrystallized from abs. methanol, and then from isopropyl alcohol, to give pure **7**, 625 mg (30%), m.p. 146° (dec.), $[\alpha]_{578}^{22}$ —169° (*c* 1.0, $CHCl_3$); ν_{max}^{KBr} 3350–3250 (OH), 1660 (C=O, α,β-unsaturated aldehyde), and 1615 cm^{-1} (C=C in conjugation with aldehyde group); ^{1}H-n.m.r. data (pyridine-d_5): δ 5.83 (t, 1 H, vinyl), 6.58 (s, broad, 3 H, 3 OH), and 9.07 (s, 1 H, CHO).

Anal. Calc. for $C_7H_{10}O_5$: C, 48.28; H, 5.79. Found: C, 48.50; H, 5.63.

*1,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-3-deoxy-*D-lyxo*-hept-2-enitol* (**9**). — Compound **7** (1.74 g, 10 mmol) was dissolved in water (20 mL) and reduced with sodium borohydride (0.2 g). After 1 h, disodium hydrogenphosphate (0.5 g) was added, and the excess of reductant was decomposed with acetic acid (~3 mL). The solution was evaporated at 40° under diminished pressure, the residue extracted with methanol (~50 mL), the extract evaporated to dryness, and the resulting syrup acetylated with pyridine (19 mL) and acetic anhydride (19 mL). The mixture was kept for 6 h at room temperature, and evaporated *in vacuo* (finally with repeated additions of toluene), to yield a semi-solid material which was extracted with boiling ether. Evaporation of the extract gave a syrup that distilled at ~180° (bath temp.)/0.05 torr, to give homogeneous (t.l.c.) **9**; yield: 3.1 g (90%), $[\alpha]_{578}^{22}$ —36.0° (*c* 1.0, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 2.05 and 2.14 (2 s, 12 H, 4 OAc).

Anal. Calc. for $C_{15}H_{20}O_9$: C, 52.33; H, 5.85. Found: C, 52.21; H, 5.97.

*2,6-Anhydro-3-deoxy-*D-lyxo*-hept-2-enitol* (**8**). — Compound **9** (3.45 g, 10 mmol) was deacetylated in 0.02M methanolic sodium methoxide solution (20 mL), and the mixture was processed as described for compound **7**. Compound **8** crystallized from ethanol; yield: 405 mg (23%), m.p. 112.8°, $[\alpha]_{578}^{22}$ +2.0° (*c* 1.0, H_2O); ν_{max}^{KBr} 3200 (OH) and 1645 cm^{-1} (C=C); ^{1}H-n.m.r. data (pyridine-d_5): δ 4.9 (s, 1 H, vinyl) and 5.9–6.5 (broad s, 4 H, 4 OH).

Anal. Calc. for $C_7H_{12}O_5$: C, 47.73; H, 6.87. Found: C, 47.86; H, 7.04.

Enzymic investigations. — β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (~10 mg) from *E. coli*, as a suspension of crystals in saturated, aqueous ammonium sulfate solution, was centrifuged, the supernatant liquor was discarded, the precipitate was taken up in sodium phosphate buffer (0.05M, pH 6.8), and the solution was dialyzed twice at 0° against 1-L portions of 0.05M sodium phosphate buffer (pH 6.8). The specific activity of the enzyme solution was ~7800 U/mL, the units (U) of enzymic activity relating to the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (2.66 mmol), which was monitored spectrophotometrically at 405 nm. [The unit (U) of enzyme is defined as that amount which catalyzes the formation of one μmol of *o*-nitrophenol per min.]

*Investigation of substrate properties of 2,6-anhydro-3-deoxy-*D-lyxo*-hept-2-enose* (**7**) *and 2,6-anhydro-3-deoxy-*D-lyxo*-hept-2-enitol* (**8**). — Solutions of **7** (50 μL, 570 mM) and **8** (50 μL, 575 mM), respectively, in 0.05M sodium phosphate buffer (pH 6.8) were treated in the presence of $MgCl_2$ (~1 mM) with β-D-galactosidase (11 U in 5 μL) at 37°. Aliquots (2 μL) were taken at 5, 10, 30, and 60 min, and 2, 4,

8, and 24 h, and then at 24-h intervals for 7 days, and examined by t.l.c. As the reference, a solution without β-D-galactosidase was used.

ACKNOWLEDGMENTS

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