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ATTEMPTED AFFINITY-LABELLING OF β-D-GALACTOSIDASE FROM *Escherichia coli* WITH 2,6:3,4-DIANHYDRO-1-DEOXY-D-*talo*-HEPT-1-ENITOL

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ABSTRACT

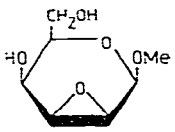
The epoxides methyl 2,3-anhydro-β-D-talopyranoside (**1**) and 2,6:3,4-dianhydro-1-deoxy-D-*talo*-hept-1-enitol (**2**), both prepared by improved methods, and 2,6:3,4-dianhydro-1-deoxy-D-*gulo*-hept-1-enitol (**3**) were applied as potential reagents for the affinity labelling of *E. coli* β-D-galactosidase. Compounds **1** and **3** are ineffective as labelling reagents, whereas compound **2** irreversibly inhibits the enzyme activity. Deactivation is complete only when high concentrations (0.5M) of the inhibitor are applied over a relatively long period of time (24 h). Saturation kinetics cannot be observed. Nevertheless, the competitive inhibitor isopropyl 1-thio-β-D-galactopyranoside protects the enzyme from irreversible deactivation by **2**, indicating that the latter also reacts with the active site. Treatment of β-D-galactosidase with 2,6:3,4-dianhydro-1-deoxy-D-*talo*-[4-^{3}H]hept-1-enitol under conditions that effect deactivation of the enzyme to only a minor extent causes labelling of the protein at the molar ratio of 48:1. Specific, radioaffinity labelling of the active site of the enzyme cannot be thus achieved.

INTRODUCTION

Several models for the catalytic action of β-D-galactosidase (EC 3.2.1.23) from *Escherichia coli* have been put forward in the literature[1,2]. Two of them consider that there is present a nucleophilic group, B, which either displaces the aglycon, with formation of a covalent galactosyl–enzyme complex (covalent catalysis), or stabilizes a previously formed, D-galactosyl cation in an ion-pair, D-galactosyl–enzyme complex (electrostatic catalysis). Both versions can be modified by assuming participation of the 2-hydroxyl group in the substrate molecule[3]. In each case, a potentially nucleophilic group B would be situated in the neighborhood of C-1 and C-2 of an enzyme-bound β-D-galactopyranoside. We now describe our attempts to modify group B by affinity labelling.

RESULTS AND DISCUSSION

Sterically suitable epoxides have frequently been used as electrophilic reagents for the affinity labelling of enzymes[4]. One of the best known examples[5] is the covalent labelling of β-D-glucosidase from *Aspergillus wentii* with one enantiomer exclusively of conduritol-B epoxide (1-D-1,2-anhydro-*myo*-inositol).



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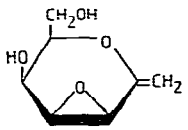

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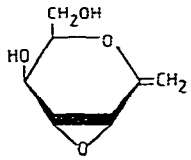

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Three epoxides (**1**, **2**, and **3**) were applied for the affinity labelling of β-D-galactosidase from *E. coli*. Methyl 2,3-anhydro-β-D-talopyranoside[6] (**1**), prepared by an improved method[7], has the closest resemblance to a β-D-galactopyranoside, but the lowest reactivity and regioselectivity for the specific labelling of group B. 2,6:3,4-Dianhydro-1-deoxy-D-*talo*-hept-1-enitol[7,8] (**2**) and -D-*gulo*-hept-1-enitol[8,9] (**3**) are structurally less related, but show a pronounced reactivity towards nucleophiles owing to their vinyl oxirane grouping[7,8,10]. If affinity to the active site of the enzyme

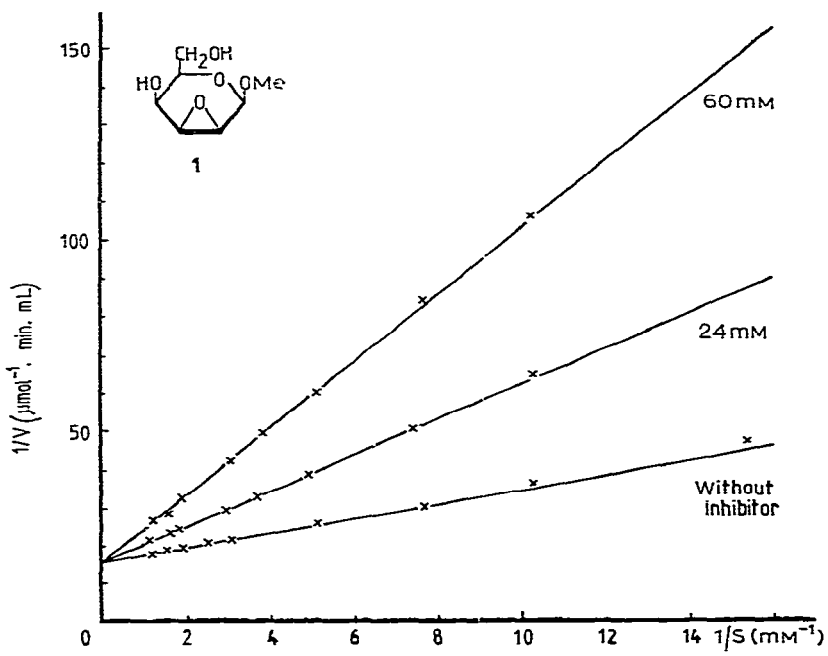


Fig. 1. Determination of the inhibition constant (K_i) for methyl 2,3-anhydro-β-D-talopyranoside (**1**) in 50mM sodium phosphate buffer (pH 6.8), containing 1mM magnesium chloride, at 30°. [The concentrations of substrate *o*-nitrophenyl β-D-galactopyranoside and inhibitor were varied, as indicated. The reaction was started by adding ~0.08 U of β-D-galactosidase per mL.]

is sufficient, covalent labelling ought to occur when the nucleophilic group is properly oriented towards the electrophilic, C-3 atom of either compound **2** or **3**.

Interaction of **1** *with the enzyme.* — Compound **1** was incubated for 48 h at room temperature with concentrations of β-D-galactosidase that would cause complete hydrolysis of methyl β-D-galactopyranoside within minutes. No change in **1** was observed, and the compound could be isolated intact from the mixture. The enzyme that had been treated with a high concentration (0.5M) of **1** retained, after dialysis, its full activity towards *o*-nitrophenyl β-D-galactopyranoside. Compound **1** is, however, a reasonable, competitive inhibitor of β-D-galactosidase action, as could be demonstrated by the Lineweaver–Burk (see Fig. 1) and Hofstee–Eadie methods. The inhibition constant (K_i) is 17mM, as compared to a K_M of ~8mM for methyl β-D-galactopyranoside[11]. These results indicate that, although **1** has an affinity to the active site of the enzyme that is not much less than that of the corresponding methyl β-D-galactopyranoside, it is, nevertheless, not hydrolyzed by the enzyme, nor does it form a covalent link by reacting therewith.

Interaction of **2** *with the enzyme.* — Compound **2** is stable in aqueous buffer solutions, and only reacts with regioselective ring-opening when such nucleophiles as azide and cyanide are present[7,8]. Just as **1** is not hydrolyzed (unlike a β-D-galactopyranoside) by β-D-galactosidase, similarly **2** is not susceptible to enzyme-catalyzed hydration of the double bond [unlike its analog 2,6-anhydro-1-deoxy-D-*galacto*-hept-1-enitol[12] (**4**)]. Here again, the inhibition constant K_i of 21mM for **2** (see Fig. 2) is comparable to the K_M of 50–70mM for the *galacto* analog[12] **4**. Prolonged incubation of

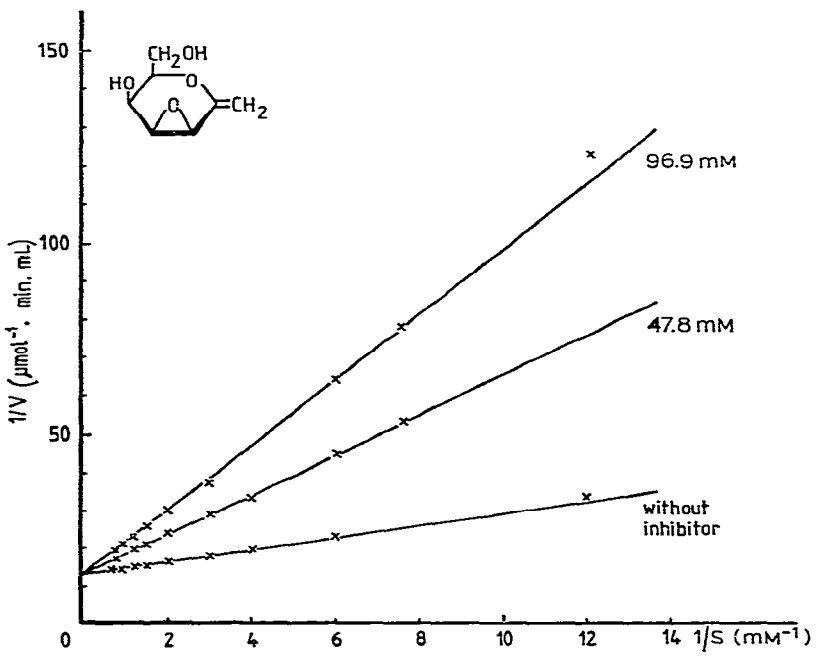


Fig. 2. Determination of the inhibition constant (K_i) for 2,6:3,4-dianhydro-1-deoxy-D-*talo*-hept-1-enitol (**2**). (For conditions see legend to Fig. 1.)

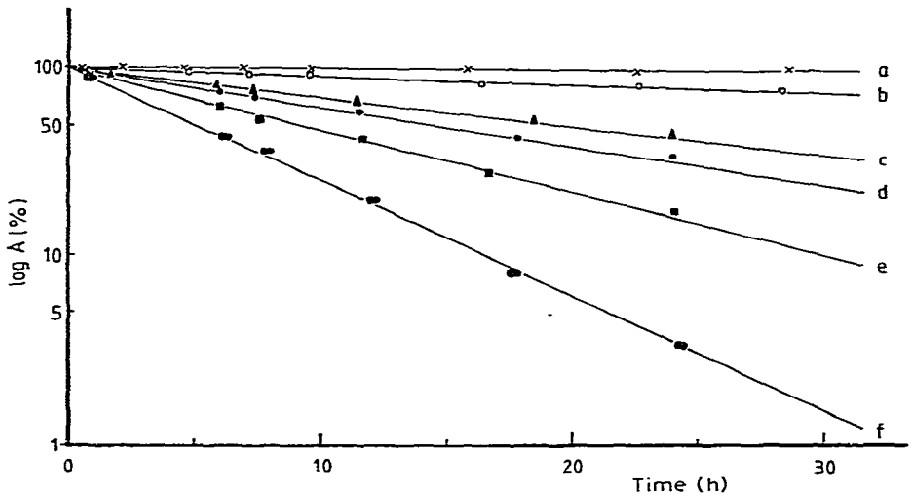
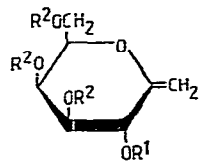


Fig. 3. Deactivation of β-D-galactosidase with 2,6:3,4-dianhydro-1-deoxy-D-*talo*-hept-1-enitol (2). [The concentrations of inhibitor were varied as indicated. The open circles give corresponding experiments with the reversible inhibitor IPTG (~60 mM). As the reference, a solution without inhibitor 2 was used (control). Key: a, ×—× control; b, ○—○ 305mM 2 in the presence of IPTG (60mM); c, ▲—▲ 136mM 2; d, ●—● 225mM 2; e, ■—■ 383 mM 2; and f, ●●—●● 585mM 2.]



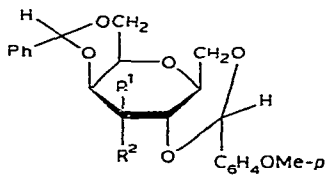
4 $R^1 = H$, $R^2 = H$
14 $R^1 = Ts$, $R^2 = Ac$

β-D-galactosidase with high concentrations (0.2–0.5M) of **2** caused irreversible inhibition of the enzyme (see Fig. 3), complete deactivation being reached after 24 h with 0.5M concentration of inhibitor. During this time, the loss of enzyme activity in a control experiment remained <5%. Enzyme activity could not be restored by dialysis of the protein, once it had been deactivated by **2**. The deactivation does not reach the point where it follows pseudo-first-order kinetics, although complete saturation of the active site ought to be reached (K_i = 21mM; inhibitor concentration = 500mM). Thus, one criterion for true affinity-labelling is not given. However, the fact that irreversible deactivation can be clearly inhibited by isopropyl 1-thio-β-D-galactopyranoside (IPTG) (see Fig. 3) is a good indication that deactivation is caused, at least partly, by labelling of the binding site of the substrate.

Interaction of **3** *with the enzyme.* — Compound **3** was available in only small quantities[7-9] (~150 mg), and therefore a proper, kinetic evaluation could not be made. There is, nevertheless, no doubt that, although having the same chemical reactivity as **2**, **3** does not deactivate β-D-galactosidase under the conditions described for the application of compound **2**.

Tracer experiments with [4-^3H]-labelled **2**. — The most rigid criterion for the evaluation of specificity is the stoichiometry of affinity labelling carried out with a radioactive reagent.

Preparation and application of 2,6:3,4-dianhydro-1-deoxy-D-talo-[*4-^3H*]*hept-1-enitol* (**2a**)*. — A potential precursor for the synthesis of [4-^{3}H]-labelled **2** (**2a**) is 2,6-anhydro-1,3-*O*-*p*-anisylidene-5,7-*O*-benzylidene-D-*glycero*-L-*manno*-heptitol[13] (**5**). The free hydroxyl group in compound **5** can be readily oxidized with Me_2SO–acetic anhydride to yield ~70% of the D-*gulo*-4-heptulose (**6**) as its hydrate (**7**), and, as a side product, the 4-(methylthio)methyl ether (**8**). Compound **7** could be dehydrated under high vacuum (over P_2O_5), to give the parent heptulose **6**, recognized by its carbonyl absorption at 1760 cm^{-1}. Reduction of **7** with sodium borohydride or lithium aluminum hydride, as well as by catalytic hydrogenation with Raney nickel, was stereospecific, and yielded **5** exclusively[7]. The ^{1}H-n.m.r. spectrum of its

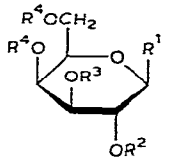


5 R^1 = OH, R^2 = H
6 R^1 . R^2 = O
7 R^1 = R^2 = OH
8 R^1 = OCH_2—SMe , R^2 = H
9 R^1 = OAc , R^2 = H

acetylated derivative (**9**) shows the signal of H-4 at δ 5.03 as a doublet of doublets, with $J_{3,4}$ 10.5 and $J_{4,5}$ 3.6 Hz, which clearly indicates the axial orientation of H-4. The corresponding reduction of **7** in 1,4-dioxane with sodium [^{3}H]-borohydride was conducted, to give **5a**, and, after acetylation, **9a**, in excellent yield. Compound **9a** was partially hydrolyzed to 2.6-anhydro-4-*O*-acetyl-5,7-*O*-benzylidene-D-*glycero*-L-*manno*-[4-^{3}H]heptitol (**10a**), as described[13] for the corresponding unlabelled compound **9**, yielding **10**. As demonstrated by ^{1}H-n.m.r. spectroscopy, the 4-*O*-acetyl group of compound **10** is stable in absolute pyridine, but migrates to the free, primary position when the substrate is kept in moist pyridine[7]. Therefore, tosylation of **10** had to be conducted in rigorously dried pyridine in order to ensure the maximal yield of radiolabelled product.

Compound **10** was converted into the 1,3-ditosylate **11**; this was hydrolyzed, and the product acetylated, to give **12**. Compound **12** underwent iodine exchange when treated with sodium iodide in boiling acetic anhydride, to yield **13**, which could be converted into the tosylate **14**, and this into **2** under the conditions described for the formation of **2** from the previously prepared mesylate[8].

*All [4-^{3}H]-labelled compounds herein will be denoted by the suffix "a".



10 $R^1 = CH_2OH, R^2 = H, R^3 = Ac, R^4, R^4 = >CH-Ph$
11 $R^1 = CH_2OTs, R^2 = Ts, R^3 = Ac, R^4, R^4 = >CH-Ph$
12 $R^1 = CH_2OTs, R^2 = Ts, R^3 = R^4 = Ac$
13 $R^1 = CH_2I, R^2 = Ts, R^3 = R^4 = Ac$
$Ts = SO_2C_6H_4-Me-p$

Compound **2a** was similarly prepared, starting from **10a**. After dilution with pure, crystalline **2**, the ^3H-labelled compound **2a** was corecrystallized three times. The sample used for labelling the enzyme protein had a specific radioactivity of 23 $mCi.mmol^{-1}$.

Compound **2a** (0.08M) was incubated for three days with β-D-galactosidase. After this time, the enzyme had retained 93% of its activity, and acquired 2.95 μCi of radioactivity per mg of exhaustively dialyzed, denatured, and washed protein. Assuming a molecular weight for tetrameric β-D-galactosidase of 464,000, 48 mol of **2a** per mol of enzyme had been incorporated by covalent binding. As the enzyme activity is scarcely affected by the concentrations of inhibitor applied, it has to be assumed that compound **2** reacts preferentially with accessible, nucleophilic groups of the protein that are not involved in the catalytic process. Owing to its high, non-specific reactivity and low affinity, **2a** is not a suitable reagent for the radio-affinity labelling of β-D-galactosidase. Active-site labelling by sufficiently high concentrations of **2a** would also cause undesirable, strong, random labelling.

EXPERIMENTAL

General methods. — Melting points were determined with a Kofler hot-stage (Bock), and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter. Infrared spectra were recorded with a Perkin–Elmer Infracord Model 137, ^1H-n.m.r. spectra with a Varian EM 390 (90 MHz) spectrometer, using tetramethylsilane or sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate as the internal standard, and $CDCl_3$, or D_2O as the solvent, unless stated otherwise. All reactions were monitored by t.l.c. on silica gel 60 F_{254} (Merck), using *A*, 4:1 (v/v) ether–light petroleum (b.p. 60–70°), or *B*, 4:1 (v/v) benzene–methanol as solvents, unless specified otherwise. Compounds were detected either by exposure to u.v. light, or by charring with sulfuric acid. Radioactive compounds on chromatograms were located with a Packard 7200 radio-chromatogram scanner. Radioactive solutions were assayed with a Multi-user liquid scintillation counter, System 8000 BF 815 (Berthold). The following "cocktails" were used: *A*, a toluene cocktail of 2,5-

diphenyloxazole (PPO, 5 g), 2,2′-*p*-phenylenebis(5-phenyloxazole) (POPOP, 62.5 mg), and toluene (1 L); *B*, a toluene–Triton cocktail of PPO (12 g), POPOP (150 mg), toluene (2 L), and Triton-X-100 (Roth; 1 L). Protein precipitates were dissolved in Protosol (New England Nuclear)–water, and then counted in cocktail *A*, or *B*.

The enzyme. — β-D-Galactosidase from *Escherichia coli* (β-D-galactoside galactohydrolase, EC 3.2.1.23) was purchased from Boehringer (Mannheim) as a suspension of crystals (5 mg/mL; specific activity, 30 U/mg) in saturated, aqueous ammonium sulfate solution. Highly purified β-D-galactosidase from *E. coli*, which was used for the inactivation of enzyme with [4-^{3}H]-labelled **2** (**2a**), was a gift from Dr. W. Littke (Freiburg); it had a specific activity of 242 U/mg. Before use, each of the two enzyme preparations was centrifuged, the precipitates were taken up in buffer (see later), and the solutions dialyzed three times against 500-mL portions of buffer. All dialyses were conducted at 4° against buffer, in dialysis tubing from Serva (Heidelberg).

Enzyme assay. — All enzyme assays were performed at 30° in 50mM sodium phosphate buffer (pH 6.8) containing 1mM magnesium chloride and *o*-nitrophenyl β-D-galactopyranoside (0.8 g/L). Release of *o*-nitrophenol was monitored in an Eppendorf photometer at 405 nm. Enzyme activity is expressed in μmol of *o*-nitrophenol produced in 1 min by 1 mg of protein under the conditions of the assay. Protein concentration was determined by the biuret procedure[14], with crystalline, bovine serum albumin as the standard. Inactivation of enzyme with different concentrations of **1**, **2**, and **3** was conducted in buffer at 30°. Aliquots (50 μL) were taken at various times, and assayed for activity in 2.5-mL portions of the incubation mixture. At this dilution, no inactivation occurs during the elapsed time of the assay.

*2,6-Anhydro-1,3-*O-p-*anisylidene-5,7-*O-*benzylidene-4-*O-*(methylthio)methyl-*D-glycero-L-manno-*heptitol* (**8**) *and 2,6-anhydro-1,3-*O-p-*anisylidene-5,7-*O-*benzylidene-*D-gulo-*4-heptulose hydrate* (**7**). — A solution of 2,6-anhydro-1,3-*O-p*-anisylidene-5,7-*O*-benzylidene-D-*glycero*-L-*manno*-heptitol[13] (**5**) (698 mg, 1.7 mmol) in dry dimethyl sulfoxide (3 mL) and freshly distilled acetic anhydride (3.5 mL) was kept at 35°, and the progress of the reaction was monitored by t.l.c. (solvent *B*). After 48 h, the starting material had been converted into two compounds (R_F 0.80 and 0.73). The mixture was poured into water (100 mL), and the precipitate was collected, washed with water, and dried *in vacuo* over phosphorus pentaoxide. The solid, dissolved in methanol, crystallized partly, to give compound **8** (116 mg, 14%); it was recrystallized from methanol. The mother liquors were evaporated to dryness, and **7** (497 mg, 69%) crystallized from 1:2 (v/v) acetone–water.

Compound **8** had m.p. 192°, $[\alpha]_{578}^{22}$ +52° (*c* 0.6, chloroform); R_F 0.80; ^{1}H-n.m.r. data: δ 2.2 (s, 3 H, S-CH_3), 3.63 (m, 1 H, H-6), 3.9 (s, 3 H, OCH_3), 3.47–4.55 (m, 8 H), 5.03 (s, 2 H, S-CH_2), 5.73 (s, 2 H, 2 CH), and 7.0–7.22 and 7.88–7.92 (2 m, 9 H, C_6H_5, C_6H_4).

Anal. Calc. for $C_{24}H_{28}O_7S$: C, 62.59; H, 6.13; S, 6.96. Found: C, 62.55; H, 6.03; S, 6.82.

Compound **7** had m.p. 165–167°, $[\alpha]_{578}^{21}$ +93° (*c* 1.0, methanol); R_F 0.73; ν_{max}^{KBr}

3350 cm^{-1} (OH); ^{1}H-n.m.r. data: δ 3.4 (s, 1 H, OH), 3.57 (s, 1 H, OH), 3.78 (s, 3 H, OCH_3), 3.67–4.42 (m, 8 H). 5.53 and 5.58 (2 s, each 1 H, 2 CH), and 6.77–7.65 (m, 9 H, C_6H_5, C_6H_4).

Anal. Calc. for $C_{22}H_{24}O_8$: C, 63.45; H, 5.81. Found: C, 63.59; H, 5.65.

*2,6-Anhydro-1,3-*O-p-*anisylidene-5,7-*O-*benzylidene-*D-gulo-*4-heptulose* (**6**) could be obtained from **7** by drying *in vacuo* at 90° over phosphorus pentaoxide; m.p. 147°; ν_{max}^{KBr} 1760 cm^{-1} (C=O).

*4-*O-*Acetyl-2,6-anhydro-5,7-*O-*benzylidene-1,3-di-*O-p-*tolylsulfonyl-*D-glycero-L-manno-*heptitol* (**11**). — A solution of 4-*O*-acetyl-2,6-anhydro-5,7-*O*-benzylidene-D-*glycero*-L-*manno*-heptitol[13] (**10**; 2.5 g, 7.7 mmol) was treated with *p*-toluenesulfonyl chloride (5 g, 26 mmol) in pyridine (15 mL), to give, after the usual processing, a brown syrup which, after dissolution in methanol, was decolorized with charcoal. After filtration, and evaporation of the filtrate, **11** was obtained as a dry foam (4.1 g, 84%), $[\alpha]_{578}^{22}$ +34° (*c* 0.95, chloroform); ^{1}H-n.m.r. data: δ 1.85 (s, 3 H, $COCH_3$), 2.33 and 2.4 (2 s, each 3 H, 2 CH_3), 3.33 (bs, 1 H, H-6), 3.5–3.83 (m, 1 H, H-2), 3.85–4.43 (m, 5 H, H-1,1',5,7,7'), 4.73–5.08 (m, 2 H, H-3,4), 5.4 (s, 1 H, CH), and 7.1–7.9 (m, 13 H, C_6H_5, 2 C_6H_4).

Anal. Calc. for $C_{30}H_{32}O_{11}S_2$: C, 56.95; H, 5.10; S, 10.13. Found: C, 56.37; H, 4.90; S, 9.92.

*4,5,7-Tri-*O-*acetyl-2,6-anhydro-1,3-di-*O-p-*tolylsulfonyl-*D-glycero-L-manno-*heptitol* (**12**). — Compound **11** (4.5 g, 7.1 mmol) was treated with 80% aqueous acetic acid (20 mL) for 2 h at 90°, and then the solution was evaporated *in vacuo*, with successive addition of water and toluene. The oily residue was acetylated with acetic anhydride (15 mL) in pyridine (15 mL), and the mixture was kept for 3 h at room temperature to give, after the usual processing, **12** as a colorless syrup (3.89, 87%), $[\alpha]_{578}^{22}$ −5.5° (*c*, 0.85, chloroform); ^{1}H-n.m.r. data: δ 1.80, 1.97, and 2.07 (3 s, each 3 H, 3 $COCH_3$), 2.4 (s, 6 H, 2 CH_3), 3.5–4.42 (m, 6 H, H-1,1',2,6,7,7'), 4.7 (t, 1 H, H-3, $J_{2,3} = J_{3,4} = 10$ Hz), 5.03 (dd, 1 H, H-4, $J_{4,5}$ 3.5 Hz), 5.33 (d, 1 H, H-5), and 7.17–7.9 (m, 8 H, 2 C_6H_4).

*4,5,7-Tri-*O-*acetyl-2,6-anhydro-1-deoxy-1-iodo-3-*O-p-*tolylsulfonyl-*D-glycero-L-manno-*heptitol* (**13**). — A solution of **12** (3.8 g, 6.04 mmol) in acetic anhydride (50 mL) containing sodium iodide (1.3 g, 8.6 mmol) was heated for 15 min at 120°. The mixture was allowed to cool to room temperature, and diluted with acetone (30 mL), and the suspension filtered with suction. The precipitate was washed with acetone, and the filtrate and washings were combined, and evaporated to a red syrup which was dissolved in dichloromethane. The solution was successively washed with water (twice) and aqueous sodium hydrogencarbonate containing a trace of sodium hydrogensulfite (to remove iodine from the organic layer), dried (magnesium sulfate), and evaporated to dryness, to give **13** as a light-yellow syrup (3.01 g, 83%), $[\alpha]_{578}^{22}$ −3.8° (*c* 0.73, chloroform); ^{1}H-n.m.r. data: δ 1.78, 2.02, and 2.13 (3 s, each 3 H, $COCH_3$), 2.42 (s, 3 H, CH_3), 3.00–3.65 (m, 3 H, H-1,1',2), 3.8–4.33 (m, 3 H, H-6,7,7'), 4.75 (dd, 1 H, H-3, $J_{2,3}$ 8.7, $J_{3,4}$ 9.5 Hz), 5.07 (dd, 1 H, H-4, $J_{4,5}$ 3.5 Hz), 5.37 (bd, 1 H, H-5), and 7.23–7.9 (m, 4 H, C_6H_4).

Compound **2** was prepared from **13** in the same way as from 4,5,7-tri-*O*-acetyl-2,6-anhydro-1-deoxy-1-iodo-3-*O*-(methylsulfonyl)-D-*glycero*-L-*manno*-heptitol[7,8]. It crystallized from acetone, and was recrystallized from ethyl acetate; m.p. 123°, $[\alpha]_{578}^{22}$ +55.5° (*c* 0.52, acetone); 250-MHz, ^{1}H-n.m.r. data ($CDCl_3/CDCl_3$, D_2O): δ 2.66 (d, 1 H, OH-5, $J_{5,OH}$ 10.5 Hz), 3.59–3.67 (m, 2 H), 3.76–3.97 (m, 3 H), 4.09 (m, 1 H, H-5, $J_{4,5}$ 5.5, $J_{5,6}$ 3 Hz), 4.81 (d, 1 H, H-1', $J_{1,1'}$ ~1.5 Hz), 4.93 (d, 1 H, H-1); (D_2O): δ 3.74–3.88 (m, 5 H), 4.24 (dd, 1 H, H-5, $J_{4,5}$ 5, $J_{5,6}$ ~3.5 Hz), 4.93 (d, 1 H, H-1, $J_{1,1'}$ 1.5 Hz), and 4.97 (d, 1 H, H-1').

Anal. Calc. for $C_7H_{10}O_4$: C, 53.16; H, 6.37. Found: C, 53.31; H, 6.40.

2,6:3,4-Dianhydro-1-deoxy-D-talo-[*4-*^{3}*H*]*hept-1-enitol* (**2a**). — To a solution of **7** (44 mg, 0.11 mmol) in hot 1,4-dioxane (1 mL) was added sodium [^{3}H]borohydride (100 mCi, sp. act. 5 Ci/mmol) at room temperature. After 12 h, the excess of **7** was reduced with sodium borohydride (t.l.c., *B*). The mixture was made neutral with glacial acetic acid, and treated with pyridine (5 mL) and acetic anhydride (5 mL) in the cold. After 12 h, ice water (2 mL) was added, and the mixture was extracted with dichloromethane (6 × 10 mL). The extracts were combined, successively washed with a saturated solution of sodium hydrogencarbonate and water, and evaporated *in vacuo*, the last traces of pyridine being removed by codistillation with toluene; this was necessary, in order to avoid acetyl migration when removing the anisylidene group in the next step. The residue was taken up in acetone (4 mL), and the solution heated with 80% aqueous acetic acid (4 mL) for 5 min at 90°; t.l.c. in solvent *B* then indicated loss of the anisylidene group. The solution was evaporated *in vacuo*, the residue treated with a solution (4 mL) of *p*-toluenesulfonyl chloride (3 g) in dry pyridine (10 mL), and kept at room temperature until the reaction was complete (t.l.c., *B*), which required ~24 h. The excess of the reagent was decomposed with water, and the product extracted with dichloromethane (5 × 5 mL); the extracts were combined, successively washed with saturated sodium hydrogencarbonate solution (2 × 10 mL) and water (10 mL), evaporated *in vacuo*, and traces of solvent codistilled with toluene. The residue was treated with 80% aqueous acetic acid (4 mL) for 4 h at 90° (t.l.c., solvent *B*), and the mixture was cooled, and evaporated *in vacuo*, to yield an oily residue which was acetylated with acetic anhydride (4 mL) in pyridine (4 mL). After 12 h, the mixture was evaporated *in vacuo*, traces of solvents being removed by codistillation with toluene, to give **12a**; this was dissolved in acetic anhydride (4 mL) containing sodium iodide (10 mg), and stirred for 15 min at 120°. T.l.c. (solvent *A*) then indicated the formation of **13a**. The colored solution was evaporated *in vacuo*, and the residue partitioned between water and dichloromethane. The organic layer was successively washed with water (2 × 50 mL), saturated sodium hydrogencarbonate solution (50 mL) containing sodium hydrogensulfite, and water (2 × 20 mL); evaporation, followed by codistillation of traces of solvent with toluene, gave a colorless syrup. A solution of this syrup in dry pyridine (2 mL) was shaken at room temperature with silver fluoride (~20 mg) until t.l.c. (solvent *A*) (detection of [4-^{3}H]**14** by spraying with dilute, aqueous potassium permanganate) indicated complete conversion within 3 h. Dichloromethane (10 mL) was then added to the

mixture, and shaking was continued for 15 min. Removal of silver salts was achieved by filtering the slurry through a Pasteur pipet filled with tissue paper. The filtrate was successively washed with 1% aqueous sodium thiosulfate (10 mL) and water (10 mL), and evaporated *in vacuo*, to give **14a**. To a solution of **14a** in acetone (4 mL) was added 0.01M sodium methoxide until the solution became alkaline, the formation of allyl epoxide **2a** being monitored by t.l.c. (detection by spraying with half-concentrated, aqueous sulfuric acid). After complete conversion of **14a** into **2a**, the solution was evaporated *in vacuo*, and the yellow residue extracted with small portions of ethyl acetate until the extract was no longer radioactive. Evaporation of the combined extracts *in vacuo* yielded a light-yellow syrup. Unlabelled **2** (20.46 mg) was added, and the mixture was dissolved, with warming, in a very small volume of ethyl acetate. After 2 d at room temperature and 1 d at −20°, the crystals of **2a** were collected on a Hirsch funnel, washed with ice-cold ethyl acetate, and recrystallized once from ethyl acetate and then once from dry acetone. Thoroughly dried (high vacuum) **2a** (19 mg) had a specific activity of 23 mCi/mmol. The combined mother liquors and washings contained more **2a**. The overall yield of **2a** was 7.5%, relative to sodium [^{3}H]borohydride.

Incubation of β-D-galactosidase with [4-^3H]2. — Pure β-D-galactosidase (4.16 mg) in buffer (0.8 mL) was added to **2a** (10.21 mg, 65 μmol, 1.49 mCi, 23 mCi/mmol). After 3 d at 26°, 93% of the enzyme was still active. The incubation mixture was dialyzed exhaustively against buffer, and the dialyzed protein contained 1.368 Ci/mmol [LSC, cocktail *A*, Protosol (0.5 mL), and water (0.1 mL); cocktail *B* and water (0.6 mL)].

Determination of (**2a**) *covalently linked to protein.* — β-D-Galactosidase modified by **2a** (10 μL, 39 μg) in buffer (190 μL) was denatured with aqueous, 40% trichloroacetic acid (100 μL) by shaking for 5 min. The suspension was centrifuged, and the radioactivity of the supernatant liquor determined [LSC, cocktail *B*, and water (0.4 mL)]. The precipitate was resuspended in buffer (200 μL) and aqueous, 40% trichloroacetic acid (100 μL), shaken for 5 min, and centrifuged. Again, the radioactivity of the supernatant liquor was determined. This procedure was repeated until no radioactivity above background level could be detected in the supernatant liquor (10 times). Finally, the radioactivity of the protein was assayed [LSC, cocktail *A*, Protosol (0.5 mL), and water (0.1 mL)]. In three, parallel determinations a proportion of ~82% (81.4, 82.0, and 82.3%) of covalently linked **2a** was found.

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REFERENCES

1 K. Wallenfels and R. Weil, *The Enzymes*, 7 (1972) 617–663.
2 M. L. Sinnott, *FEBS Lett.*, 94 (1978) 1–9.

3 M. BROCKHAUS, H.-M. DETTINGER, G. KURZ, J. LEHMANN, AND K. WALLENFELS, *Carbohydr. Res.*, 69 (1979) 264–268.
4 D. MERCIER, A. OLESKER, S. D. GERO, AND J. E. G. BARNETT, *Carbohydr. Res.*, 18 (1971) 227–231; J. C. MILLER AND S. G. WALEY, *Biochem. J.*, 123 (1971) 163–170; E. M. BESSELL AND J. H. WESTWOOD, *Carbohydr. Res.*, 25 (1972) 11–21; K. J. SCHRAY, E. L. O'CONNELL, AND I. A. ROSE, *J. Biol. Chem.*, 248 (1973) 2214–2224; E. L. O'CONNELL AND I. A. ROSE, *ibid.*, 248 (1973) 2225–2231; G. LEGLER AND E. BAUSE, *Carbohydr. Res.*, 28 (1973) 45–52; M. L. SHULMAN, S. D. SHIYAN, AND A. YA. KHORLIN, *Biochim. Biophys. Acta*, 445 (1976) 169–181; Y. ESHDAT AND N. SHARON, *Methods Enzymol.*, 46 (1977) 403–414.
5 H. BRAUN, G. LEGLER, J. DESHUSSES, AND G. SEMENZA, *Biochim. Biophys. Acta*, 483 (1977) 135–140.
6 L. F. WIGGINS, *J. Chem. Soc.*, (1944) 522–526.
7 B. SCHWESINGER, Ph.D. Thesis, University of Freiburg i.Br., 1981.
8 J. LEHMANN AND B. SCHWESINGER, *Chem. Ber.*, 111 (1978) 3961–3964.
9 J. LEHMANN AND B. SCHWESINGER, *Chem. Ber.*, submitted.
10 M. BROCKHAUS, W. GORATH, AND J. LEHMANN, *Justus Liebigs Ann. Chem.*, (1976) 89–96.
11 J.-P. TENU, O. M. VIRATELLE, J. GARNIER, AND J. YON, *Eur. J. Biochem.*, 20 (1971) 363–370; C. K. DE BRUYNE AND M. YDE, *Carbohydr. Res.*, 56 (1977) 153–164.
12 M. BROCKHAUS AND J. LEHMANN, *Carbohydr. Res.*, 53 (1977) 21–31.
13 J. LEHMANN AND B. SCHWESINGER, *Carbohydr. Res.*, 72 (1979) 267–271.
14 G. BEISENHERZ, H. J. BOLTZE, T. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT, AND G. PFLEIDERER, *Z. Naturforsch., Teil B*, 8 (1953) 555–577 (see p. 576).