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**Note**

# Ester and sulfonium salt formation in the active-site labeling of β-D-galactosidase from *Escherichia coli* by 2,6-anhydro-1-deoxy-1-diazo-D-*glycero*-L-*manno*-heptitol

MANFRED BROCKHAUS AND JOCHEN LEHMANN
*Chemisches Laboratorium der Universität Freiburg, Albertstr. 21, D-7800 Freiburg i.Br. (Germany)*
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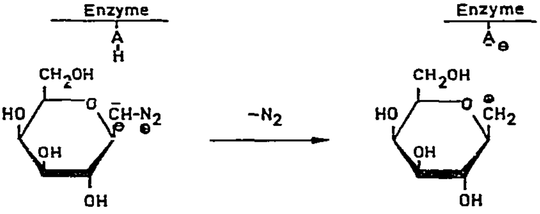
2,6-Anhydro-1-deoxy-1-diazo-D-*glycero*-L-*manno*-heptitol (**1**) is an efficient, active-site-directed, irreversible inhibitor of β-D-galactosidase from *Escherichia coli* (EC 3.2.1.23)[1]. Its effect on a hypothetical proton-donating group "AH", normally responsible for the protonation of the glycosidic oxygen atom, should be that of a suicide substrate.



1 2

Compound **1**, which bears close steric as well as polar resemblance to a β-D-galactoside, should also be bound to the enzyme in much the same way. Protonation by "AH" of its C-1 atom creates a highly reactive carbonium ion **2**. It is reasonable to assume that **2** will react with the conjugate base of "AH".

$^3$H-Labeled **1** was used to obtain radioactively labeled, inactivated β-D-galactosidase. Part of the label could be removed from the protein by mild treatment with hydroxylamine, indicating its previous attachment through an ester bond[2] (Fig. 1). This label could be recovered and identified by cocrystallization with authentic 2,6-anhydro-D-*glycero*-L-*manno*-heptitol (**3**). The residual label is resistant to hydroxylamine at 37°. It can be detached* by prolonged heating in neutral buffer (Fig. 2) and has the same chromatographic properties as 2,6-anhydro-1-deoxy-1-*S*-methyl-1-thio-D-*glycero*-L-*manno*-heptitol (**4**). After oxidation with peroxyformic acid and subsequent acetylation, the $^3$H-labeled compound crystallized with the acetylated

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*10% of the initial radioactivity remains bound to the protein.

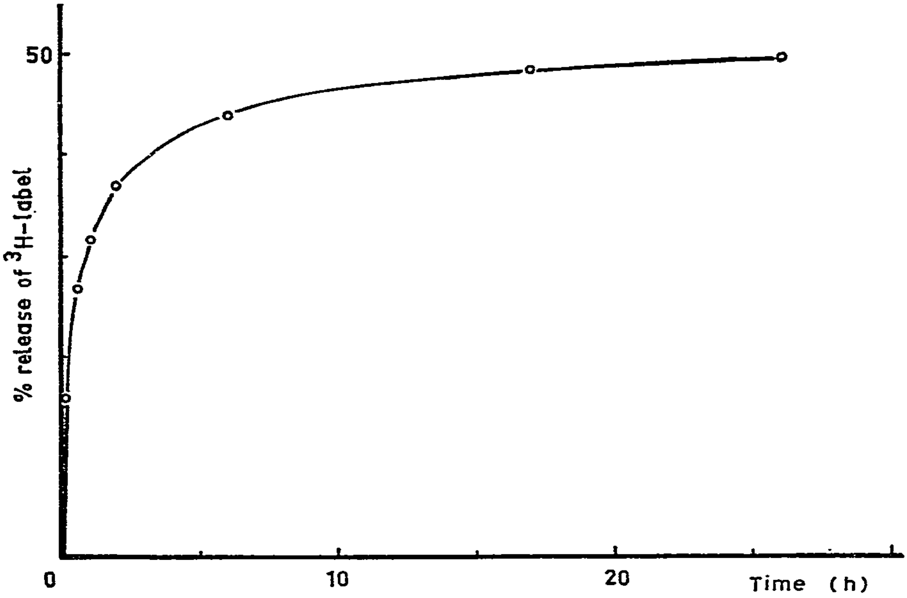
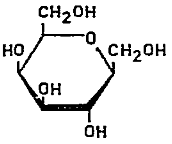


Fig. 1. Release of $^3H$ label from pH 7.0 inactivated enzyme by 0.67M hydroxylamine, pH 9.5, at 37°.

sulfone (5) of 4. These experiments proved that **1** not only reacted as expected with the acid "AH", but also with the thioether group of a methionine residue to yield a sulfonium salt. This sulfonium salt decomposes[3] on heating and releases **4**.



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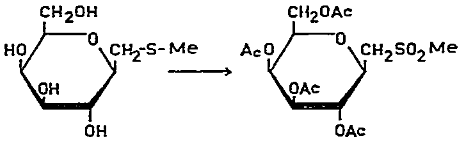

4 5

A methionine residue was also labeled in the active-site-directed inactivation of *β*-D-galactosidase with *N*-bromoacetyl-*β*-D-galactopyranosylamine[4], *N*-bromoacetyl-*β*-D-glucopyranosylamine[5], and 3-(*β*-D-galactopyranosylmethyl)-1-(*p*-nitrophenyl)triazene[6]. We suggest that methionine is not involved as a reactive group in the catalytic action of *β*-D-galactosidase. It is either situated close to the actual protonating carboxylic acid "AH", and is therefore able to compete for the carbonium ion **2** because of its superior nucleophilicity, or it may be part of another binding-site, possibly the aglycon binding-site, where it becomes alkylated independently. A second binding-site, other than the glycon binding-site, was recently discussed by Viratelle *et al.*[5]. The possibility of methionine being a part of the aglycon binding-site, where **1** may also be bound, is supported by an interesting coincidence. The ratio of methionine to carboxylate-labeling increases from 4:5 at pH 7.0 to 7:4 at pH 8.0. This change parallels the increase, with increasing pH, of the transglycosylation-to-hydrolysis ratio for lactose, as found by Huber, Kurz, and Wallenfels[7]. The latter finding suggests an increase in aglycon binding with increasing pH values.

## EXPERIMENTAL

*General methods.* — T.l.c. was performed on silica gel $F_{254}$ (Merck), and paper chromatography on Whatman No. 1 paper with 6:4:3 1-butanol–pyridine–water as solvent. G.l.c. was performed with a Pye Unicam GCD chromatograph, with glass columns and 3% SE-52 on Chromosorb G, AW-DMCS. N.m.r. data (internal $Me_4Si$) were obtained with Varian A60-D and EM 390 spectrometers, i.r. data with a Perkin–Elmer Infracord model 137 spectrometer, and optical rotations with a Perkin–Elmer 141 polarimeter. Radioactive samples were assayed with a Berthold BF 8000 liquid-scintillation counter. Radioactive chromatograms were counted in a Packard 7200 Radiochromatogram scanner.

*1-Acetamido-2,6-anhydro-*D-glycero-L-manno-[7-$^3$H]*heptitol.* — 1-Acetamido-3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-D-*glycero*-L-*manno*-heptitol[8] (27 mg) was dissolved in 10mM sodium methoxide in methanol (3 ml). After 3 h, the solution was passed through a column (1 × 10 cm) of silica gel with methanol as eluant, to remove the base. The effluent was evaporated and the residue dissolved in 10mM sodium phosphate buffer (pH 7.2). D-Galactose oxidase (EC 1.1.3.9, Sigma, 1.3 mg) and catalase (EC 1.11.1.6, Boehringer, 0.5 mg) were added. The reaction at 20° was monitored by g.l.c. of (trimethylsilyl)ated[9] samples. After 6 h, the solution was concentrated to 200 $\mu$l by lyophilization and added to a solution (50 $\mu$l) of $NaB^3H_4$ (100 mCi, 6.0 Ci/mmol, Buchler) in 10% aqueous trisodium phosphate. After 14 h, the solution was acidified with acetic acid, and evaporated, and the residue (150 mCi) was chromatographed on Whatman No. 3 paper with 248:142:200 (v,v,v) 1-butanol–propanoic acid–water as solvent. The title compound was located by autoradiography (Kodak, single coated, blue-sensitive, X-ray film, exposure time 12 h), eluted with water, and freeze dried.

*3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-(*N*-nitrosoacetamido)-*D-glycero-L-manno-[7-$^3$H]*heptitol.* — The preceding compound (10 mCi) was treated with 1:1 acetic anhydride–pyridine (200 $\mu$l) for 12 h. After evaporation under diminished pressure, the residue was stirred at 0° with chloroform (3 ml) and dinitrogen tetraoxide (50 $\mu$l). The reaction was monitored by t.l.c. with 4:1 ether–light petroleum as solvent. After 2 h, the solution was washed with saturated aqueous sodium hydrogencarbonate (5 × 10 ml) and then with water (5 × 10 ml). The organic layer was dried (magnesium sulfate) and evaporated. The residue (7.3 mCi) was dissolved in ether (100 ml), and nonradioactive title compound[1] (8 mg) was added. The product was first crystallized from ether–light petroleum and then from methanol–water to give 4 mg (395 mCi/mmol) of product.

Although the nonradioactive title compound has been described and identified in a previous paper[1] its spectral data were not published and are given here. $^1$H-N.m.r. data (60 MHz, $CDCl_3$): $\delta$ 1.98, 2.03, 2.13, and 2.20 (4s, each 3H, 4 OAc), 2.82

(s, 3H, NAc), 3.5–4.6 (m, 6H, H-1,2,6,7), 5.0–5.5 (m, 3H, H-3,4,5); $\nu_{max}^{KBr}$ 1740 (-OCO) 1710 cm$^{-1}$ (-NCO).

*Generation of* **1** in situ *and labeling of it by β*-D-*galactosidase*. — 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-(*N*-nitrosoacetamido-D-*glycero*-L-*manno*-[7-$^3$H]-heptitol (2.4 mg, 395 mCi/mmol) was dissolved in 0.1M methanolic sodium methoxide (50 μl). Conversion into **1** was complete after 2 min, as shown by i.r. (4.8 μm)[1] and u.v. spectra ($\varepsilon_{max}$ 435 nm)[11]. The solution was then added portionwise (10 μl) to a solution of β-D-galactosidase (1.3 mg) in 0.2M Tris–HCl pH 7.0 or 8.0 (200 μl) that was mM with respect to magnesium chloride. The solutions were then dialyzed at 4° against mM sodium phosphate buffer (pH 7.0) until no further radioactivity was detectable in the outer compartment.

*Liberation of* $^3$*H-label from β*-D-*galactosidase*. — To 500 μl of a solution of labeled enzyme (0.1 mg, 1.3 × 10$^6$ *d.p.m.*) were added 500 μl of bovine serum albumin (2.5 mg, Boehringer) in water and 500 μl of 2M aqueous hydroxylamine, pH 9.5 (ref. 2). Aliquots (50 μl) were removed and treated with 25 μl of 50% trichloroacetic acid for 5 min at room temperature. After centrifugation, the radioactivity was determined in the supernatant liquid (see Fig. 1). After exhaustive treatment with hydroxylamine, the residual protein solution (1 ml) was treated with 50% trichloroacetic acid (500 μl). The precipitate was washed with water (2 × 1 ml) and suspended in 0.05M sodium phosphate (pH 6.5, 500 μl). The suspension was divided into 10 equal portions, each of which was heated to 95°. At suitable intervals, the portions were treated with 50% trichloroacetic acid (25 μl) for 5 min at room temperature and the mixtures were centrifuged. The radioactivity in the supernatant solution and in the precipitate (solubilized in 75 μl of 70% formic acid) was determined separately (see Fig. 2).

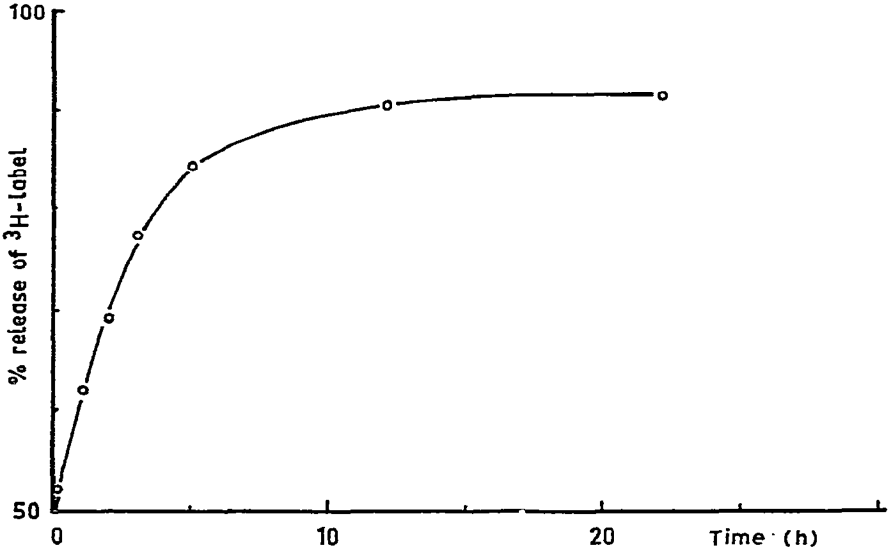

Fig. 2. Release of $^3$H label from pH 7.0 inactivated enzyme at 95° and pH 6.5.

*Identification of $^3$H label as 2,6-anhydro-*D-glycero-L-manno-*heptitol* (3). — A solution of hydroxylamine-treated protein (50 μg, 660,000 d.p.m.) was denatured and centrifuged. The protein-free supernatant solution was freed from trichloroacetic acid and hydroxylamine by subsequent elution from ion-exchange columns (10 ml of Amberlite IRA-400 and 3 ml of Amberlite IR-120). The radioactivity in the eluate cochromatographed exclusively with **3**. The eluate (250,000 d.p.m.)* was mixed with **3** (100 mg) and freeze dried. The residue was crystallized from ethanol to constant radioactivity (d.p.m./mg, no. of crystallizations given): 2370, 1; 2160, 2; 2176, 3; and 2220, 4.

*Identification of $^3$H label as 2,6-anhydro-1-deoxy-1-*S*-methyl-1-thio-*D-glycero-L-manno-*heptitol* (4) *and 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-(methylsulfonyl)-*D-glycero-L-manno-*heptitol* (5). — When 2-mercaptoethanol was added for protection against oxidation, the radioactivity in the protein-free solution of the experiment corresponding to Fig. 2 co-chromatographed exclusively with **4**. The solution (104,000 d.p.m.) was freeze dried, and the residue was dissolved in formic acid (100 μl) and 30% hydrogen peroxide (100 μl), and stored at room temperature for 6 h. Acetic anhydride–pyridine (2 ml) was added, and the mixture, after keeping for 12 h at room temperature, was evaporated *in vacuo*. The residue was dissolved in chloroform (3 ml) and washed with water (2 × 3 ml). The organic layer was dried (magnesium sulfate) and evaporated. The residue (39,500 d.p.m.)* was mixed with 110 mg of **5** and crystallized from 2-propanol to constant radioactivity (d.p.m./mg, no. of crystallizations given): 338, 1; 296, 2; 286, 3; 280, 4; and 282, 5.

*3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-*S*-methyl-1-thio-*D-glycero-L-manno-*heptitol.* — 2,6-Anhydro-1-*O*-tosyl-D-*glycero*-L-*manno*-heptitol[10] (0.96 g) was dissolved in abs. *N,N*-dimethylformamide (10 ml) and stirred for 12 h at room temperature with sodium methanethioxide (2.0 g). Pyridine–acetic anhydride (1:1, 50 ml) was added, the mixture was heated for 1 h at 100°, and evaporated under diminished pressure. The residue was dissolved in chloroform (40 ml) and washed with water (2 × 40 ml). The organic layer was dried (magnesium sulfate) and evaporated to a syrup (950 mg) that could be distilled at 190° $10^{-3}$ torr, yield: 930 mg (86%); $[\alpha]_{578}^{25}$ +5.4° (*c* 1.0, chloroform); n.m.r. data (60 MHz, $CDCl_3$): δ 1.98, 2.15, and 2.20 (3s, each 3H, 3 OAc), 2.05 (s, 6H, OAc and $SCH_3$), 2.67 (d, 2H, $CH_2S$, $J_{1,1}$ 5.5 Hz), 3.55–4.30 (m, 4H, H-2,6,7), and 5.0–5.5 (m, 3H, H-3–5).

*Anal.* Calc. for $C_{16}H_{24}O_9S$: C, 48.97; H, 6.16. Found: C, 49.45; H, 6.36.

*3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-methylsulfonyl-*D-glycero-L-manno-*heptitol* (5). — To a solution of 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-*S*-methyl-1-thio-D-*glycero*-L-*manno*-heptitol (930 mg) in formic acid (5 ml) was added 30% aqueous hydrogen peroxide (1 ml). The mixture was kept for 1 h and processed by adding chloroform (50 ml) and washing the organic layer with saturated aqueous sodium hydrogencarbonate (2 × 100 ml) and with water (50 ml). After drying (magnesium sulfate) and evaporation, the product was crystallized from

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*Loss of radioactivity by chemical processes could not be avoided.

2-propanol to give 790 mg (78%) of 5; m.p. 167–170°, $[\alpha]_{578}^{25}$ +19.0° (*c* 1.0, chloroform); $\nu_{\max}^{\mathrm{KBr}}$ 1750 (CO), 1610 ($SO_2$), and 1260 cm$^{-1}$ ($SO_2$); n.m.r. data (90 MHz, $CDCl_3$): $\delta$ 1.92, 1.97, 2.00, and 2.12 (4s, each 3H, 4 OAc), 2.97 (s, 3H, $SO_2CH_3$), 2.9–3.5 (m, 2H, H-1), 3.9–4.2 (m, 4H, H-2,6,7), and 5.0–5.5 (m, 3H, H-3–5).

*Anal.* Calc. for $C_{16}H_{24}O_{11}S$: C, 45.28; H, 5.70; S, 7.55. Found: C, 45.03; H, 5.75; S, 7.55.

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