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SYNTHESIS OF 2,6-ANHYDRO-*S*-[ETHYLMERCURY(II)]-1-THIO-D-*glycero*-L-*manno*-HEPTITOL AND BIS(2,6-ANHYDRO-1-THIO-D-*glycero*-L-*manno*-HEPTITOL)MERCURY(II), AND THE STUDY OF THEIR INTERACTION WITH β-D-GALACTOSIDASE FROM *E. coli**

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ABSTRACT

Two competitive inhibitors of β-D-galactosidase activity, namely, 2,6-anhydro-*S*-[ethylmercury(II)]-1-thio-D-*glycero*-L-*manno*-heptitol (**4**) and bis(2,6-anhydro-1-thio-D-*glycero*-L-*manno*-heptitol)mercury(II) (**6**), with inhibition constants of 8.0×10^{-4}M and 1.9×10^{-4}M, were synthesized. Compound **6** was incorporated into the crystalline enzyme by cocrystallization. The stoichiometry of the enzyme–inhibitor complex was 1:4, corresponding to one molecule of inhibitor per active site of the enzyme. Compound **4** was found to be unstable against X-ray irradiation, whereas compound **6** was submitted to X-rays for several days without any radiation damage.

INTRODUCTION

β-D-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23) has been, and still is, one of the key proteins in modern genetics[1], as well as one of the most intensively investigated enzymes. The three-dimensional structure is, however, still unknown, the main reason being lack of large enough single crystals. This problem has recently been overcome[2] and thereby the basis laid for successful structural investigations[3].

In order to localize the four active sites of β-D-galactosidase, special heavy-metal-containing, competitive inhibitors which could be used as isomorphic replacement for phase determination were needed. Such compounds have to fulfil several requirements: they have to be (*a*) chemically stable under normal conditions (aqueous solutions, room temperature, *etc.*) and (*b*) soluble in water. (*c*) The molecular bulk must not exceed that of an ordinary substrate. (*d*) They must not be hydrolyzed by the enzyme. (*e*) The inhibition constant must be suitably low. (*f*) The heavy metal must combine high mass number with low valency.

*Dedicated to Professor Arthur Lüttringhaus on the occasion of his 80th birthday.

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(*g*) The compound in question has to be stable under prolonged X-ray irradiation. We now describe a class of mercury-organic compounds which fulfil the aforementioned requirements.

RESULTS AND DISCUSSION

Because, of all the heavy-metal atoms, only mercury forms organic compounds which possess the stability specifications, we attempted to synthesize D-galactopyranosyl derivatives containing this element.

CHEMICAL SYNTHESES

Competitive, hydrolysis-resistant inhibitors of β-D-galactosidase are normally 1-thio-β-D-galactopyranosides. They combine good affinity and also chemical inertness in the presence of β-D-galactosidase in high concentrations. The easiest way in which to prepare a 1-thio-β-D-galactopyranoside containing organomercury seemed to be by causing direct reaction of a suitable sulfhydryl derivative with an alkylmercury halide. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-galactopyranose (**1**) in acetone reacted satisfactorily with ethylmercury chloride, to give 2,3,4,6-tetra-*O*-acetyl-*S*-[ethylmercury(II)]-1-thio-β-D-galactopyranoside (**2**). *O*-Deacetylation by the Zemplén procedure led to gradual decomposition, with liberation of metallic mercury. A small amount of deacetylation product isolated by chromatography on silica gel decomposed rapidly when dissolved in water. Changing the nature of the substituent R, as well as changing the conditions of deacetylation, were of no avail. The apparent high reactivity in protic solvents of a sulfur-mercury substituent attached to the anomeric carbon atom, already observed for D-glucosyl compounds by Ferrier and Furneaux[4], makes components of this type unsuitable for our purpose.

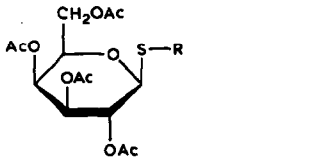
Compared with 1-thio-β-D-galactopyranosides[5] (see Table I), the equally enzyme-resistant β-D-galactopyranosyl derivatives 2,6-anhydro-D-*glycero*-L-*manno*-heptitol (**3**) K_I = 19mM (refs. 6 and 7), 2,6-anhydro-3-deoxy-*aldehydo*-D-

TABLE I

K_I VALUES FOR SEVERAL β-D-GALACTOPYRANOSYL DERIVATIVES

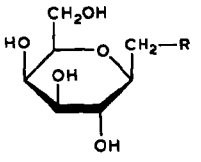
Compound	K_I (mM)
Isopropyl 1-thio-β-D-galactopyranoside	0.085
2-Hydroxyethyl 1-thio-β-D-galactopyranoside	0.32
Phenyl 1-thio-β-D-galactopyranoside	0.19
o-Nitrophenyl 1-thio-β-D-galactopyranoside	0.3
2,6-Anhydro-D-*glycero*-L-*manno*-heptitol	19.0
2,6-Anhydro-*S*-[ethylmercury(II)]-1-thio-D-*glycero*-L-*manno*-heptitol	0.8
Bis(2,6-anhydro-1-thio-D-*glycero*-L-*manno*-heptitol)mercury(II)	0.19

lyxo-hept-2-enose, K_I = 11mM (ref. 8), or 2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enitol, K_I = 22mM (ref. 8), where the pyranosyl system is attached to a carbon atom (*C*-glycosyl derivatives), have rather poor affinities for the binding sites of β-D-galactosidase, and yet the absence of an anomeric hetero-atom, as in [*S*-ethylmercury(II)]-1-thio-β-D-galactopyranoside, which is susceptible to decomposition, promised higher chemical stability in protic solvents. 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-iodo-D-*glycero*-L-*manno*-heptitol[9] gave 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-*S*-benzoyl-1-thio-D-*glycero*-L-*manno*-heptitol (**5**) in almost quantitative yield when treated with sodium thiobenzoate in boiling DMF. When **5** and ethylmercury chloride were added in equimolar amounts to methanolic sodium methoxide, formation of 2,6-anhydro-[*S*-ethylmercury(II)]-1-thio-D-*glycero*-L-*manno*-heptitol (**4**) took place. When, instead of ethylmercury chloride, mercury acetate was used, the product was bis(2,6-anhydro-1-thio-D-*glycero*-L-*manno*-heptitol)mercury(II) (**6**). When sufficiently purified by recrystallization, both com-



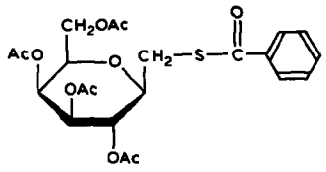
1 R = H

2 R = Hg—CH_2CH_3



3 R = OH

4 R = S—Hg—CH_2CH_3



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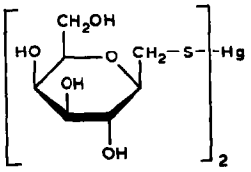

6

pounds can be stored at room temperature without any sign of decomposition. They are soluble in water, and are not hydrolyzed at room temperature.

Enzymic investigations. — Both compounds **4** and **6** inhibited the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (*o*NPGal) by β-D-galactosidase at quite a low concentration. The inhibition constants were determined by Lineweaver–Burk plotting, $K_I = 8.0 \times 10^{-4}$M for compound **4**, and $K_I = 1.9 \times 10^{-4}$M for **6** (see Figs. 1 and 2). An irreversible inhibition of the enzyme by **4** and **6** could not be observed; this was determined in the following way. The enzyme was incubated with **4** and **6** for several h at 4°. Enzyme assay as described in the legend to Fig. 1 was, however, carried out by starting with addition of the substrate. No difference in enzyme assay could be found, regardless of whether the enzyme was preincubated with the inhibitor or not.

In the presence of **6**, β-D-galactosidase can be crystallized on a preparative

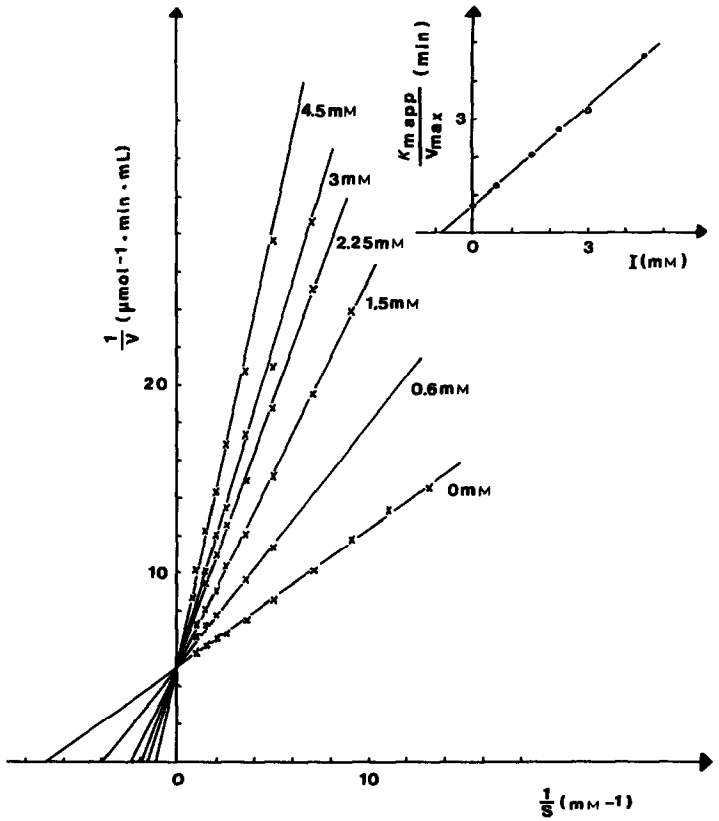

Fig. 1. Reciprocal plot for different fixed concentrations of compound 4. [Insert shows secondary plot of the slope of each reciprocal plot *versus* the corresponding inhibitor concentration. The intercept on the [I] axis gives $-K_I$. (β-D-Galactosidase, as crystal suspension in ammonium sulfate solution, was centrifuged, the supernatant liquor discarded, and the residue dissolved in sodium potassium phosphate buffer (0.05M, pH 6.8), containing (mM) magnesium chloride. Remaining ammonium sulfate was removed by Sephadex G 25 chromatography, using the buffer system as eluant. The activity was determined with *o*NPGal (2.66 mM; 30°). The hydrolysis was monitored spectrophotometrically at 405 nm. The protein concentration was determined on the basis of its optical absorbance at 280 nm. The specific activity of the enzyme was 394 U/mg. One incubation was run with 0.3 U, a unit (U) being defined as the amount of enzyme liberating 1 µmol of *o*-nitrophenol from *o*NPGal/min.]

scale by adding saturated ammonium sulfate solution. Repeated washings with salt solution gave pure, crystalline material. Quantitative determination of the mercury content by atomic absorption spectroscopy, using the cold-vapor technique, indicated that, per subunit of protein, *e.g.*, per active site, one molecule of inhibitor was incorporated into the crystal. In Fig. 3, the time of successive washings (average, three times per h) is plotted against the inhibitor content of the crystalline material. Eventually, a constant content of four molecules of compound **6** per molecule of protein was reached.

Examination of single crystals under X-ray irradiation. — Single crystals of

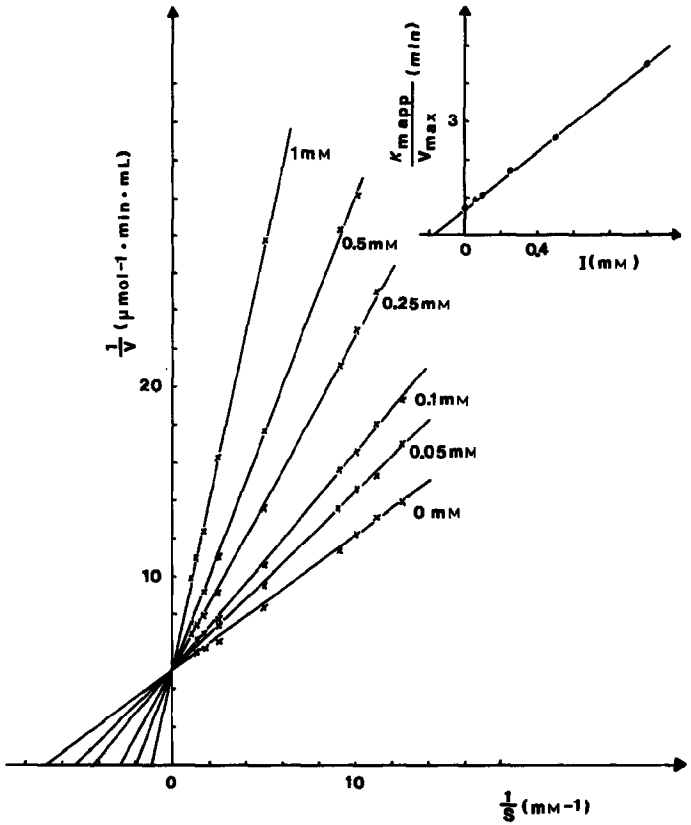

Fig. 2. Reciprocal plot for different fixed concentrations of compound **6**. Insert shows secondary plot of the slope of each reciprocal plot *versus* the corresponding inhibitor concentration. The intercept on the [I] axis gives $-K_I$. (For experimental details see Fig. 1.)

compounds **4** and **6** were grown from solution by slow evaporation. Large, but very thin, platelets of **4** were obtained from 17:2:1 (v/v/v) ethyl acetate–methanol–water, whereas **6** grew as long needles from 2:1 (v/v) ethanol–water. Suitable crystals were glued to glass fibers mounted in goniometer heads, and examined by the X-ray precession technique[10]. Under the influence of CuKα radiation, the clear, colorless crystals of **5** became a shining deep-black in color after a few minutes. At the same time, reflections became blurred and then disappeared. Compound **4**, as a single crystal, therefore proved too labile for X-ray examination.

In order to examine more closely the process of decomposition by X-rays, powdered crystals, and the corresponding saturated aqueous solution, of **4** were placed in capillary tubes and exposed for >24 h to CuKα radiation. T.l.c. analysis of the decomposition products of **4** revealed almost complete destruction of the starting material. Besides a number of minor products (not identified) the main component proved to be **6**, as unambiguously demonstrated later by isolation, and comparison with the authentic compound. Apparently, the radiation energy causes

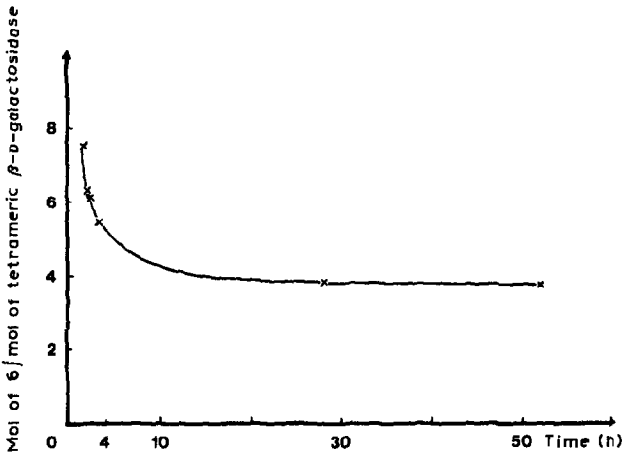

Fig. 3. Stoichiometric incorporation of compound **6** into crystalline β-D-galactosidase. [β-D-Galactosidase (7.5 mg/mL; 469 U/mg) together with compound **6** (2.273 mg/mL), corresponding to the presence of 95% enzyme-inhibitor complex E·I was crystallized at 0° by adding saturated, aqueous ammonium sulfate solution. The crystals were subsequently washed with the same ammonium sulfate solution by resuspending, centrifuging, and removing the supernatant solution. For diffusion of excess **6** from the crystals during washing procedures, 20 min were allowed. The final two washings had a diffusion time of 24 h. Quantitative mercury determination was carried out by using the mercury cold vapor technique at 200°, a wavelength of λ 253.4 nm and an internal standard. The quantitative evaporation of covalently bound mercury was proved by double determination.]

the disproportionation of **4** into the thermodynamically more stable, symmetrical product **6**. This finding adds further evidence to the rule that mercury tends to form symmetrical, rather than unsymmetrical, covalent derivatives[11]. Hardly any decomposition was found when **6** itself was treated under the aforementioned conditions. Therefore, it proved well suited for further investigation as a heavy-metal ligand for marking the active site in β-D-galactosidase.

Recent results have shown that compound **6** is not only a competitive inhibitor of β-D-galactosidase action, but also inhibits competitively with a lag-phase, catalytic activity of alpha amylase from porcine pancreas.

EXPERIMENTAL

General methods. — Melting points are uncorrected. Only p.a. solvents were used. Light petroleum refers to the fraction having b.p. 60–70°. All reactions were monitored by t.l.c. on silica gel F_{254} (Merck), using the solvents indicated. Flash chromatography[12] was performed on silica gel (230–400 mesh, Merck), with solvents as indicated. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker WM 250 (250 MHz) spectrometer, using $CDCl_3$ as the solvent (internal standard, Me_4Si).

3,4,5,7-Tetra-O-acetyl-2,6-anhydro-1-S-benzoyl-1-thio-D-glycero-L-manno-

heptitol (**5**). — 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-iodo-D-*glycero*-L-*manno*-heptitol[7] (2.5 g, 5.29 mmol) was vigorously stirred with potassium thiobenzoate (2.6 g, 14.81 mmol) in abs. *N,N*-dimethylformamide (50 mL) for 0.5 h at 150° (t.l.c. 2:3 ethyl acetate–light petroleum). After being cooled, the mixture was poured onto ice (150 mL), with stirring. The precipitate formed was filtered off, dissolved in dichloromethane (200 mL), and the solution successively washed with aqueous sodium hydrogensulfite (4%, 200 mL) and ice–water (3 × 200 mL), dried ($MgSO_4$), and evaporated to dryness *in vacuo*. The residue was chromatographed twice on a column (2 × 23 cm) of silica gel (1:3 ethyl acetate–light petroleum), to yield **4** as a colorless syrup (2.12 g, 83%); $[\alpha]_{578}^{25}$ −8.5° (*c* 1.0, chloroform); for ^{1}H-n.m.r. data, see Table II.

2,6-Anhydro-S-[*ethylmercury(II)*]-*1-thio*-D-glycero-L-manno-*heptitol* (**4**). — Compound **5** (1.0 g, 2.07 mmol) and ethylmercury chloride (0.55 g, 2.07 mmol) were dissolved in abs. methanol (250 mL), the pH was adjusted to 9.0 with 0.1M sodium methoxide, and the mixture was stirred in the dark under nitrogen and for 3 h at room temperature, the pH being repeatedly corrected (t.l.c. in 7:2:1 ethyl acetate–methanol–water). When the reaction was complete, silica gel (20 g) was added, and the mixture was swirled for 5 min. Following filtration, the filtrate was evaporated under diminished pressure. Then, the residue was digested with 2-propanol (20 mL), and the solution decanted, and evaporated to dryness. The residue was dissolved in water (3 mL) and applied to a column (1.5 × 22 cm) of silica gel. The column was first washed with 9:1 cyclohexane–acetone (190 mL) to remove the excess of ethylmercury chloride[13]. Then, 17:2:1 ethyl acetate–methanol–water was used as the solvent to elute the product. After evaporation,

TABLE II

250 MHz, ^{1}H-N.M.R. DATA[a] ($CDCl_3$, INTERNAL Me_4Si) AND ^{1}H-N.M.R. SPIN-COUPLING DATA

Proton	**5**	***4ac***	***6ac***	$J_{H,H}$ *(Hz)*	**5**	***4ac***	***6ac***
H-1	3.50	3.15	3.18	1,1′	14.55	14.55	14.55
H-1′	3.14	2.99	3.01	1,2	2.85	2.40	2.25
H-2	3.63	3.56	3.66	1′,2	8.25	7.35	6.75
H-3	5.25	5.33	5.41	2,3	9.90	10.20	10.20
H-4	5.05	5.05	5.05	3,4	9.90	10.50	10.50
H-5	5.44	5.46	5.46	4,5	3.15	3.30	3.30
H-6	3.89	3.98	3.99	5,6	1.05	1.05	0.75
H-7	4.16	4.18	4.32	6,7	6.75	7.20	6.75
H-7′	4.07	4.11	4.17	6,7′	6.75	6.30	6.75
$-COCH_3$	2.10	2.07	2.11	7,7′	12.00	10.50	11.25
$-CH_2-$	—	1.58	—				
$-CH_3$	—	1.30	—				
$-o-C_6H_5$	7.97						
$-m-C_6H_5$	7.47						
$-p-C_6H_5$	7.60						

[a]Chemical shifts (δ).

the solid residue was recrystallized twice from 7:2:1 ethyl acetate–methanol–water, giving colorless **5** (335 mg, 37%); m.p. 138°, $[\alpha]_{578}^{25}$ −14° (*c* 1.0, water); for ^{1}H-n.m.r. data, see Table II.

Anal. Calc. for $C_9H_{18}HgO_5S$: C, 24.63; H, 4.13; S, 7.31. Found: C, 24.64; H, 3.88; S, 7.12.

Bis(2,6-anhydro-1-thio-D-glycero-L-manno-*heptitol)mercury(II)* (**6**). — Compound **5** (1.5 g, 3.11 mmol) and mercury acetate (495 mg, 1.55 mmol) were dissolved in abs. methanol (750 mL), the pH was adjusted to 9.0 with 0.1M sodium methoxide, the mixture was stirred under nitrogen in the dark, the pH being repeatedly corrected (t.l.c. 7:2:1 ethyl acetate–methanol–water). When the reaction was complete, silica gel (50 g) was added, and the mixture was swirled until it was neutral. Solid material was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water (4 mL), and the solution applied to a column (2 × 20 cm) of silica gel. By-products were eluted with 17:2:1 ethyl acetate–methanol–water (300 mL), and compound **6** was then eluted with 7:2:1 ethyl acetate–methanol–water. The eluate was evaporated until the residue began to crystallize. The product solidified completely when kept for 12 h at 4°. Recrystallization from 7:2:1 ethyl acetate–methanol–water yielded **6** as colorless crystals (904 mg, 47%; m.p. 198°, $[\alpha]_{589}^{25}$ −32.6° (*c* 1.0, water); for ^{1}H.n.m.r. data, see Table II.

Anal. Calc. for $C_{14}H_{26}HgO_{10}S_2$: C, 27.16; H, 4.23; S, 10.36. Found: C, 26.38; H, 4.24; S, 10.47.

Acetylation of compounds **4** *and* **6**. — For n.m.r. spectroscopy, both compounds were acetylated. Of each, 10 mg was dissolved at 0° in abs. pyridine (1 mL), treated with acetic anhydride (0.5 mL), and kept for 24 h at room temperature in the dark (t.l.c. 2:1 ethyl acetate–light petroleum). Evaporation to dryness under a pressure of 13.3 Pa yielded a yellowish syrup which was purified on a column (0.5 × 5 cm) of silica gel with 1:1 ethyl acetate–light petroleum as the solvent. Evaporation yielded the colorless acetylation products.

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