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Note

Some synthetic inhibitors of β-D-glucosiduronase*

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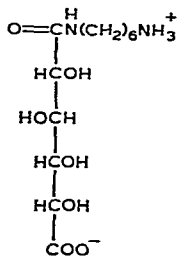
β-D-Glucosiduronase, one of the most important enzymes involved in carbohydrate metabolism, is widespread in mammalian tissues and body fluids, as well as in lower animals and bacteria[1]. This enzyme is known to occur in many forms[2–4], and is being intensively studied from the standpoint of biochemical genetics[5–8], their clearance from serum[4,9–11], and their uptake by skin fibroblasts.

Despite the importance of β-D-glucosiduronase, relatively few synthetic inhibitors have been reported. Availability of synthetic inhibitors for this enzyme not only aids in metabolic studies, but also provides tools in purifying the enzyme by affinity chromatography. Three types of inhibitor are being used for the preparation of affinity sorbents for glycosidases: 1-thioglycosides[12], aldonamides[13], and *N*-glycosyl compounds[14,15]. We now report the preparation of D-glucaro-1-[*N*-(6-aminohexyl)amide (**1**) and *N*-1-β-D-glucopyranosyl(6-aminohexanamide)uronic acid (**7**), two inhibitors of β-D-glucosiduronase belonging to the last two classes of inhibitor, for possible use in affinity sorbents.



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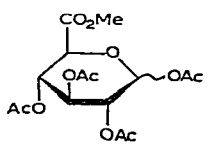

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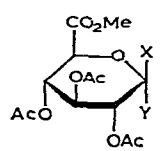
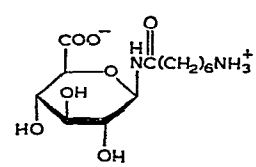


3 X = H, Y = Br
4 X = N_3, Y = H
5 X = NH_2, Y = H
6 X = $NHC(CH_2)_6NHCCF_3$, Y = H
(O O)



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EXPERIMENTAL

Materials. — The following chemical compounds were obtained commercially from the sources indicated: D-glucaro-1,4-lactone, D-glucurono-6,3-lactone, *p*-nitrophenyl β-D-glucopyranosiduronic acid, and bovine liver β-D-glucosiduronase (Sigma Chemical Co.); 1,6-hexanediamine (Aldrich Chemical Co.); platinum oxide (Ventron Alfa Products, Beverly, MA); and Rexyn 101 cation-exchange resin (200–400 mesh) (Fisher Scientific Co.). 6-(Trifluoroacetamido)hexanoic acid was prepared as described previously[16].

Methods. — All evaporations were conducted under diminished pressure below 40°. Thin-layer chromatography (t.l.c.) was performed on aluminum plates precoated with a layer of silica gel F-254 (Merck). Solvent systems used were: (*A*) 7:3 (v/v) ethanol–water, (*B*) 1:1 (v/v) benzene–ether, (*C*) 1:1 (v/v) benzene–ethyl acetate, and (*D*) 3:2:1 (v/v) ethyl acetate–acetic acid–water. T.l.c. zones were made visible by spraying with (*i*) 15% H_2SO_4 in 50% ethanol, and heating at 130° (for D-glucuronic acid derivatives), (*ii*) 4% ninhydrin in acetone, and heating (for amino-containing compounds), or (*iii*) periodate–permanganate reagent[17] (for compounds containing vicinal hydroxyl groups). Sugars were determined by a modified phenol–sulfuric acid method[18], and amino group was analyzed quantitatively by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method[18]. Melting points (uncorrected) were measured with a Fisher–Johns apparatus. Proton magnetic resonance (p.m.r.) spectra were recorded with a JEOL NMH-100 spectrometer, and optical rotation was measured with a Cary 60 spectropolarimeter. Elemental analyses were performed by Galbraith Labs, Inc. (Knoxville, Tenn.).

β-D-Glucosiduronase activity was assayed at 37° by a slight modification of the method of Fishman *et al.*[19], using *p*-nitrophenyl β-D-glucopyranosiduronic acid as the substrate. The reaction mixture comprised the following: 0.1M sodium acetate buffer, pH 4.5, containing 1mM EDTA, 0.35 mL; the substrate (0–10mM), 0.05 mL; and the inhibitor (0–1mM), 0.05 mL. To this solution was added 0.05 mL of β-D-glucosiduronase solution (1 mg/mL), and tubes were incubated for 30 min at 37°. The reaction was stopped by adding 2.5 mL of 0.25M glycine buffer, pH 10.4, containing 0.2M NaCl. The absorbance at 400 nm was measured with a Bausch and Lomb colorimeter. The rate of hydrolysis was linear, at least up to 45 min.

D-*Glucaro-1*-[N-(*6-aminohexyl*)*amide*] (**1**). — To a solution of 1,6-hexanediamine (2.32 g, 20 mmol) in dry methanol (25 mL) was added D-glucaro-1,4-lactone (1.92 g, 10 mmol) with stirring. The lactone dissolved quickly to give a yellow solution that slowly became turbid. Water was added dropwise until the turbidity cleared. The reaction was over within one hour, as judged by t.l.c. (solvent *A*; staining with the ninhydrin and with the periodate–permanganate reagents). The mixture contained, in addition to the diamine, only one component (compound **1**) positive to both ninhydrin and periodate–permanganate, and another component (D-glucaric acid) positive only to the latter reagent.

The mixture was evaporated, the residue dissolved in water, and the solution

applied to a column (2.4 × 16 cm) of Rexyn 101 resin (NH_4^+). The column was successively eluted with water and 0.5M NH_4OH, and the fractions (5 ml) were analyzed by t.l.c. (solvent *A*). The water eluate contained D-glucaric acid, and the 0.5M NH_4OH eluate contained pure product **1**; the diamine was not eluted from the column, even with M NH_4OH. Fractions containing **1** were combined, and evaporated, and the resulting, white solid was recrystallized by dissolving in water and adding 95% ethanol to slight turbidity. Crystals were thus obtained in 72% yield; m.p. 178–180°, $[\alpha]_D^{25}$ +15.24° (*c* 5.0, water); pure by t.l.c. (solvent *A*). An aqueous solution of the product is neutral, and amino group analysis by the TNBS method, using 6-aminohexanoic acid as the standard, gave 97% of the theoretical value.

Anal. Calc. for $C_{12}H_{24}N_2O_7$ (308.33): C, 46.74; H, 7.85; N, 9.09. Found: C, 46.54; H, 8.00; N. 8.89.

*Methyl (2,3,4-tri-*O*-acetyl-α-*D*-glucopyranosyl bromide)uronate* (**3**). — Methyl 1,2,3,4-tetra-*O*-acetyl-D-glucuronate (**2**) was prepared according to Bollenback *et al.*[20]. Compound **2** (20 g, 53 mmol) was stirred in 30% HBr in glacial acetic acid (65 mL) at room temperature until dissolved. The mixture was kept overnight at 4°, poured into cold chloroform (400 mL), and the solution poured into cold water (1 L) containing ice chips, with vigorous stirring. The mixture was then transferred to a separatory funnel, and the chloroform layer was separated. The aqueous layer was extracted with cold chloroform (400 mL), and the chloroform solutions were combined, washed with cold water (2 × 500 mL), dried (Na_2SO_4), and evaporated. The resulting syrup was dissolved in ether (200 mL), petroleum ether (b.p. 30–60°) was added to incipient turbidity, and the mixture kept overnight in the cold. Crystals of **3** were collected, and washed with petroleum ether; yield: 17.94 g (45 mmol, 85%), m.p. 168–173°, homogeneous by t.l.c. (solvent *B*).

*Methyl (2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosyl azide)uronate* (**4**). — To a suspension of sodium azide (4 g) in dry *N,N*-dimethylformamide (80 mL) was added a solution of **3** (5 g, 12.6 mmol) in chloroform (40 mL). The suspension was stirred overnight at room temperature, poured into cold water (500 mL), and the mixture extracted with chloroform (2 × 200 mL). The extracts were combined, successively washed with cold water (3 × 400 mL) and 0.1M NaCl (400 mL), dried (Na_2SO_4), and concentrated to ~20 mL. To this solution were added ethyl acetate (30 mL) and then petroleum ether (b.p. 30–60°) until a slight cloudiness persisted. The needle-shaped crystals that formed on standing were collected by filtration, and washed with petroleum ether. The azide (**4**) thus obtained had m.p. 152–153°, and was pure by t.l.c. (solvent *B*).

Anal. Calc. for $C_{13}H_{17}N_3O_9$ (359.29): C, 43.46; H, 4.77; N, 11.70. Found: C, 43.28; H, 4.72; N, 11.63.

*Methyl (2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosylamine)uronate* (**5**). — A solution of **4** (1.2 g, 3.34 mmol) in ethyl acetate (27 mL) and platinum oxide catalyst (0.25 g) were placed in a 100-mL, two-necked flask, and hydrogen was introduced while the suspension was being stirred magnetically. The progress of hydrogenation was monitored by t.l.c. (solvent *B*), which showed that the reaction was complete in 3.5 h.

The catalyst was filtered off, and the colorless filtrate was evaporated, to yield 1.1 g of **5** (99% yield) as a white solid, which was recrystallized from ethyl acetate–petroleum ether (b.p. 35–60°); m.p. 139–140°.

Methyl {2,3,4-tri-O-acetyl-β-D-glucopyranosyl-[6-(trifluoroacetamido)hexanamide]}uronate (**6**). — The amino group of **5** was coupled to the carboxylic group of 6-(trifluoroacetamido)hexanoic acid (**8**) with the aid of dicyclohexylcarbodiimide (DCC). The reaction mixture initially contained 0.53 g (1.6 mmol) of **5**, 0.41 g (1.8 mmol) of **8**, and 0.38 g (1.85 mmol) of DCC in dry dichloromethane (25 mL), and the reaction was monitored by t.l.c. in solvent *C*. The mixture was kept overnight at room temperature, and then the same amounts of **8** and DCC were added. After 40 h at room temperature, the reaction was 60–70% complete, as judged from t.l.c. The crystals of 1,3-dicyclohexylurea were filtered off, and the filtrate was evaporated to a syrup; addition of absolute ethanol gave **6** as white crystals, and the filtrate contained both **5** and **6**. The crystals were recrystallized from absolute ethanol (yield 42%), m.p. 158–159°.

Anal. Calc. for $C_{21}H_{29}F_3N_2O_{11}$ (542.46): C, 46.49; H, 5.39; N, 5.17. Found: C, 46.68; H, 5.35; N, 5.21.

N-1-β-D-Glucopyranosyl(6-aminohexanamide)uronic acid (**7**). — The three protective groups on **6**, *i.e.*, methyl, acetyl, and trifluoroacetyl groups, were removed in two steps. First, the *O*-acetyl groups were removed by dissolving **6** (0.3 g, 0.55 mmol) in 0.05M sodium methoxide in dry methanol (5 mL), keeping the solution

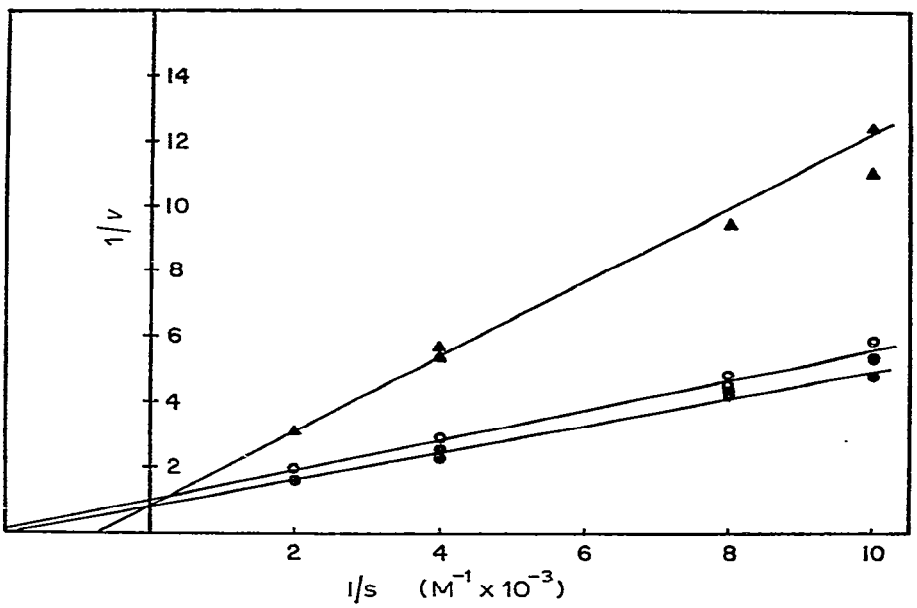

Fig. 1. Lineweaver–Burke plot of β-D-glucosiduronase hydrolysis of *p*-nitrophenyl β-D-glucopyranosiduronic acid in the presence of inhibitor **1** or **7**. (Solid circle, no inhibitor; open circle, 0.2mM **7**; triangle, 1mM **1**.)

overnight at room temperature, and evaporating to dryness. The methyl ester and *N*-trifluoroacetyl groups were then removed by treatment with aqueous alkali. The residue was dissolved in 0.1M NaOH (4 mL), and, as the hydrolysis proceeded, the pH was periodically adjusted to >11 by addition of M NaOH. After a few hours at room temperature, the mixture was kept overnight in the cold, made neutral with 5.7M HCl, concentrated to ~5 mL, and the concentrate applied to a column (2 × 143 cm) of Sephadex G-15. Elution with 0.1M acetic acid accomplished desalting, as well as removal of some minor contaminants. Fractions containing mostly **7** (t.l.c. in solvent *D*) were combined, and evaporated. The residue was dissolved in water, and 95% ethanol was added to incipient turbidity. On cooling, compound **7** crystallized as needles (yield 0.12 g, 0.39 mmol; 71%); m.p. 210° (dec.), $[\alpha]_D^{25}$ −29.6° (*c* 5.5, water). Analysis for amino group by the TNBS method, with 6-aminohexanoic acid as the standard, showed 98% of the theoretical value.

Anal. Calc. for $C_{12}H_{23}N_2O_8 \cdot 0.5\ H_2O$ (315.33): C, 45.70; H, 7.35, N, 8.88. Found: C, 45.65; H, 7.9; N, 8.66.

Inhibition of β-D-glucosiduronase. — The inhibitory power of compounds **1** and **7** was tested at 1mM (for **1** and **7**) and 0.2mM (for **7**) with various concentrations of substrate. The nature of the inhibition and its binding constant were determined by using a Lineweaver–Burke plot (see Fig. 1). Compound **1** is a competitive inhibitor, with K_i of 700 μM, whereas **7** is not a simple competitive inhibitor. Inhibition was also measured at a substrate concentration of 0.5mM and inhibitor concentrations ranging from 0 to 5mM. Fifty-percent inhibition of the initial velocity was observed at 1.2mM for **1**, and 0.6mM for **7**.

Stability of **1**. — Separate solutions of **1** in water, M acetic acid, 0.01M NaOH, and buffer solutions of pH 4.5 and 8.5 were kept for 3 days at room temperature. There was no change in the concentration of the amino group in any of the solutions (TNBS method) during this period. T.l.c. (solvent *A*) also showed no sign of breakdown of **1**.

DISCUSSION

Aldonolactones are effective inhibitors of glycosidases[21]. For β-D-glucosiduronase, D-glucaro-1,4- and -1,5-lactone are the two most powerful inhibitors known, the latter being the more powerful[22]. Unfortunately, because of its reactivity, the lactone structure would be difficult to maintain intact during chemical reactions leading to attachment to solid matrices, or during affinity chromatography itself. In this regard, Harris *et al.*[23] reported coupling of D-glucaro-1,4-lactone *via* a presumed amide bond involving C-(6)-O_2H. However, the product in this reaction scheme was not characterized. As β-D-glucose analogs of β-D-glucopyranosiduronic acids are not known to inhibit β-D-glucosiduronase, it is questionable whether the adsorbent used in this study was really based on enzymic affinity.

The effectiveness of sugar lactones as glycosidase inhibitors is believed to lie in their half-chair conformational structure, which resembles the transitional structure

believed to be assumed by glycosides during hydrolysis[21]. It is, therefore, intriguing that Kanfer *et al.*[13] used (acyclic) aldonamides effectively as affinants for purification of glycosidases. In this case also, aldonamide purportedly formed on the sorbent was not chemically identified, and no attempt was made to test for inhibitory power of presynthesized aldonamides. In our study, an aldonamide (**1**) was indeed found to be a competitive inhibitor of β-D-glucosiduronase, although its binding to the enzyme is 1/100th to 1/500th of that of the parent lactone, namely, D-glucaro-1,4-lactone.

Wolfrom *et al.*[24] reported that various aldonamides are hydrolyzed in water, the process being enhanced by the addition of alkali. We found, however, that **1**, an *N*-substituted aldonamide, is quite stable under comparable conditions, and should therefore be able to withstand most of the conditions used in affinity chromatography.

Glycosylamines are also known inhibitors of glycosidase[25], and their stable amide derivatives have been successfully used for affinity chromatography of lectins[26,27] and glycosidases[14,15]. In analogy to other glycosylamine derivatives, the D-glucopyranuronic acid counterpart (**7**) described here also proved to be an inhibitor, although its inhibitory mode was found to be more complicated than that of a simple competitive inhibitor. These compounds may become useful in studies related to β-D-glucosiduronase, especially in affinity chromatography of the enzyme.

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