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Inhibition of some hepatic lysosomal glycosidases by ethanolamines and phenyl 6-deoxy-6-(morpholin-4-yl)-β-D-glucopyranoside

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Abstract

The hepatic lysosomal glycosidases α-glucosidase and β-glucuronidase were inhibited in vitro and in vivo by mono- and diethanolamines. The in vivo inhibition is dose dependent and occurs at a value less than LD_{50}. Phenyl 6-deoxy-6-(morpholin-4-yl)-β-D-glucopyranoside inhibited α-glucosidase both in vitro and in vivo. The treatment of the enzymes in vitro by ethanolamine exhibited a reversible inhibition of the mixed and competitive types for α-glucosidase and β-glucuronidase, respectively. Diethanolamine showed a reversible inhibition of the competitive type for both enzymes. It is a potent inhibitor for β-glucuronidase, in vitro, whose inhibition constant (K_i) is 5×10^{-5} M. Phenyl 6-deoxy-6-(morpholin-4-yl)-β-D-glucopyranoside is a potent inhibitor only for hepatic α-glucosidase with a K_i value of 1.6×10^{-5} M. The pattern of the pH dependence of enzymic activity was not affected by ethanolamine inhibition. The magnitude of the inhibition of enzymes is dependent on the structure of the inhibitor.

Keywords: α-Glucosidase; β-Glucosidase; β-Glucuronidase; Morpholine; Glucopyranoside; Inhibitors

1. Introduction

Carbohydrate-processing enzymes and, in particular, glycosidases [1–3] play various roles in important biological processes. Modifying or blocking these processes for therapeutic or biotechnological applications with the help of potent and selective inhibitors for these enzymes has become an attractive target. Lysosomal α-glucosidase (α-D-glucoside glucohyrolase, EC 3.2.1.20) is an exoglucosidase that has both α-(1→4)- and α-(1→6)-glucosidase activities. This enzyme plays an important role in glycogen breakdown by catalyzing the hydrolysis of lysosomal glycogen [4]. Many mammalian acid α-glucosidases have been purified from different sources [5]. Acid α-glucosidases possess both maltase and glycoamylase activities [6], and these have pH optima in the range of pH 4–5 [6–16]. Another important lysosomal glycosidase is β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31), which hydrolyzes the glucuronide bond at the nonreducing termini of glycosaminoglycans [17]. The level of this enzyme activity is elevated in some human

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tumors [18–20]. It has been reported that acidic α-glucosidases are regulated within the regulation of lysosomal glycogen metabolism [5], whereas β-glucuronidase is regulated by phosphorylation of its carbohydrate [20] or protein [21] moieties.

Glycosidase inhibitors exert their biological effect by competitively inhibiting specific glycoside-processing enzymes, and much attention has been directed to developing therapeutic agents acting on such enzymes whereby some potential beneficial drugs are clinically evaluated [22,23]. 1-Deoxynojirimycin (**1**, DNM, moranoline) is a potent inhibitor for all types of mammalian α-glucosidases, and has anti-HIV activity [24]. The respective derivative 1,5-dideoxy-1,5-[(6-deoxy-1-*O*-methyl-6-α-D-glucopyranosyl)imino]-D-glucitol (MDL 73945) is a selective and potent intestinal α-glucohydrolase inhibitor [25]. Examination of the skeleton of 1-deoxynojirimycin and its analogues shows that the β-hydroxyalkylamine residue can be considered a part of such inhibitors. The skeleton of MDL 73945 (**2**) has the nitrogen linked to the C-6 of a 6-deoxyglucoside. Since there is still a need for structural analogues of DNM [2,3,26], we designed glycosidase inhibitors having these structural features by considering the disconnection in **1**, whereby ethanolamine (**4**) and diethanolamine (**5**) will be our targets. Replacing the 1-deoxynojirimycin ring in **2** by a simple cyclic amine ring having a chair conformation and characterized by a close similarity to the DNM moiety led to **3**, whose inhibition constant (K_i) was found to be 1.3×10^{-4}. Although this value is low, it is still in the range of consideration [27] against β-glucosidase from sweet almond. Consequently, in this paper, the respective phenyl glucoside analogue **6** will be our target (Fig. 1).

2. Results and discussion

The target compounds **4** and **5** are commercially available, whereas **6** was prepared by selective tosylation of phenyl β-D-glucopyranoside (**7**) to afford the 6-*O*-tosyl derivative **8** [28]. Nucleophilic displacement of the tosyloxy group in **8** with morpholine gave phenyl 6-deoxy-6-(morpholin-4-yl)-β-D-glucopyranoside (**6**), whose acetylation with acetic anhydride in pyridine gave phenyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-(morpholin-4-yl)-β-D-glucopyranoside (**9**) (Scheme 1). The structure of **9** was confirmed from studying its ^1H, ^{13}C and 2D NMR spectra. The ^1H NMR spectra indicated the presence of three acetyl groups and protons corresponding to the morpholinyl ring. The $^1H-^1H$ D QFCOSY experiment of **9**

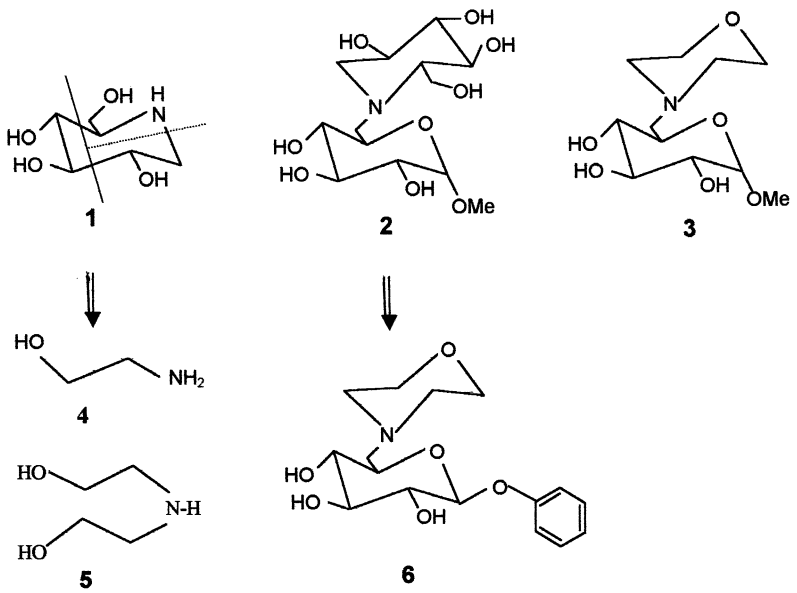
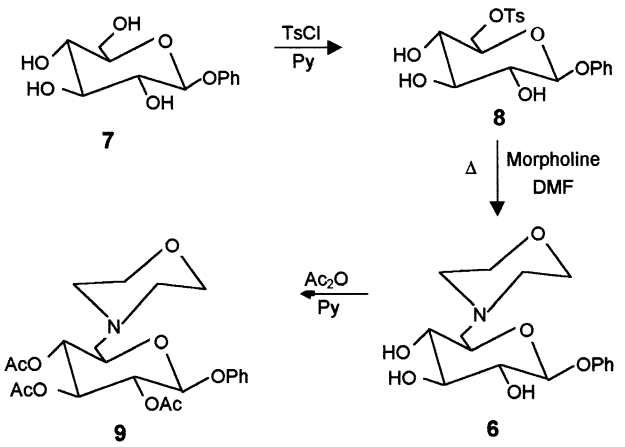


Fig. 1. Structural formulae for compounds **1**–**6**.



Scheme 1.

facilitated the correlation of protons with each other and helped in the assignment of the sugar protons. The $^{1}H-^{13}C$ HMQC experiment helped in identifying the carbon atoms of the sugar moiety.

In vitro inhibition of α-glucosidase and β-glucuronidase.—When the purified enzymes were assayed in the absence and presence of different amounts of ethanolamines, specific activities were shown to be inhibited in a concentration-dependent manner. The relative specific activities (ratio of the specific activity of **4**- or **5**-treated enzyme to that of the control) of both enzymes were decreased on increasing the amounts of **4** (Fig. 2(A)) and **5** (Fig. 2(C)). The IC_{50} values of **4** were 26.0 and 42.5 μg, and those of **5** were 33.0 and 19.0 μg for α-glucosidase and β-glucuronidase, respectively. This inhibition implies that there is a direct interaction between the tested enzymes and ethanolamines. The reversibility of inhibitor binding was readily realized when, after incubation for 60 min with **4** and **5**, full activity of the enzymes was recovered following dialysis for 16 h at 4 °C. Moreover, increasing the time of incubation to 12 h did not affect the recovery of the enzyme activity compared to that of the control. When the purified enzymes were assayed in the absence and presence of different amounts of **6**, the specific activity of α-glucosidase was shown to be inhibited in a concentration-dependent manner. The relative specific activity of α-glucosidase was decreased on increasing the concentration of **6** (Fig. 3(A)). The value of the IC_{50} of **6** was found to be 13.0 μg. On the other hand, the relative specific activity of β-glucuronidase was not significantly changed by **6**.

In vivo inhibition of α-glucosidase and β-glucuronidase.—The measured LD_{50} for each compound is calculated from the dose that is lethal to half of the treated mice. This value is 30 mg (1.5 mg/g body wt) and 50 mg (2.5 mg/g body wt) for **4** and **5**, respectively. Specific activities of the examined lysosomal glycosidases from mice treated with **4** and **5** decreased in a dose-dependent manner, compared with those of controls from vehicle-treated mice. α-Glucosidase and β-glucuronidase from mice treated with **4** (1.5 mg/g body wt) displayed relative activity values of 21 and 45%, respectively (Fig. 2(B and D)). This means that α-glucosidase showed a 5-fold decrease in relative activity compared with the control at that dose. According to the values of the specific activity, this decrease is significant ($P < 0.01$). At a dose equal to 60% of LD_{50} (18 mg/g body wt), α-glucosidase and β-glucuronidase displayed relative activities of 76.0 ($P < 0.30$) and 60.6 ($P < 0.05$)%, respectively. Therefore, β-glucuronidase is the enzyme that is significantly inhibited at a dose far from LD_{50}, and **5** has a more significant inhibitory effect than **4**.

The specific activity of α-glucosidase from mice treated with **6** decreased to 77% by 3.25 mg (0.16 mg/g body wt) as compared with the control (Fig. 3(B)). This means that α-glucosidase showed a 1.28-fold decrease in the relative specific activity. β-Glucuronidase was not affected by **6** as compared with that of vehicle-treated mice.

Kinetic studies of in vitro treated enzymes.—In order to study the type of in vitro inhibition of both α-glucosidase and β-glucuronidase and measure the K_i values, Dixon plots of enzymatic reactions were constructed by increasing the concentrations of **4** and **5**. For the inhibition of α-glucosidase by **4**, the plot of $1/v_0$ versus **4** yielded straight lines that intersect at the y-axis (Fig. 4(A)). This pattern is characteristic of mixed-type inhibition [29]. A K_i value of 3.5×10^{-4} M was obtained. However, Lineweaver–Burk analysis of α-glucosidase inhibition by **4** gave straight lines that intersect to the left of the y-axis, confirming the results (data are not

shown). On the other hand, a Dixon plot of the inhibition of α-glucosidase by **5** and β-glucuronidase by **4** and **5** yielded straight lines that intersect to the left of the y-axis (Fig. 4(B–D)), a pattern that is characteristic of competitive inhibition [29].

The K_i value of α-glucosidase inhibition by **5** is 1.3×10^{-4} M, while those of β-glucuronidase inhibition by **4** and **5** were 2×10^{-4} and 5×10^{-5} M, respectively. Therefore, the degree of inhibition by **5** is higher for β-glucuronidase than that of α-glucosidase. The Dixon plot of the inhibition of α-glucosidase by **6** (Fig. 4(E)) yielded straight lines which intersect at the x-axis. This pattern is characteristic of noncompetitive inhibition, and the K_i value is 1.6×10^{-5} M.

It was noticed that the data of time course of both enzymes are important through measuring the initial velocity values (v_0) for mak-

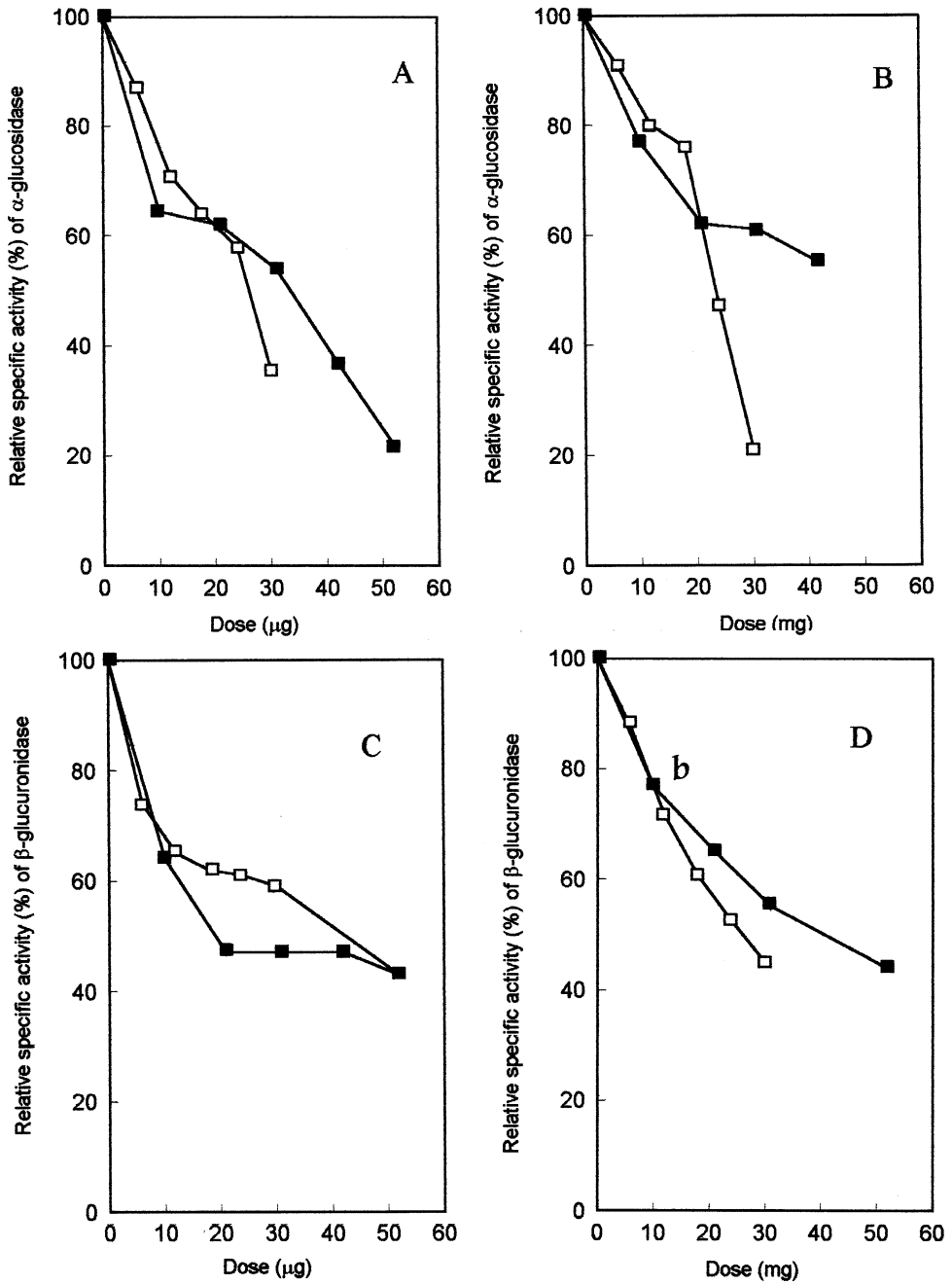


Fig. 2. Effect of ethanolamines on α-glucosidase and β-glucuronidase. (A and C) in vitro treatments; (B and D) in vivo treatments. (□) **4**; (■) **5** at 37 °C.

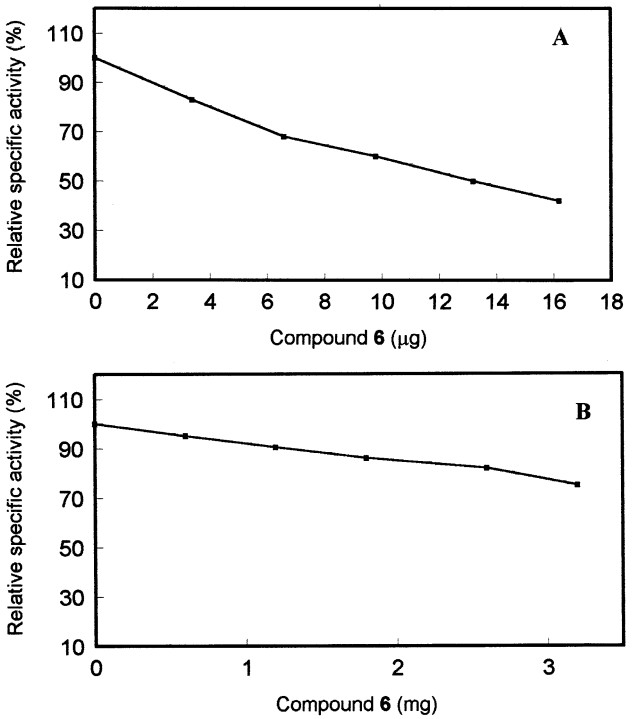


Fig. 3. Effect of **6** on α-glucosidase in vitro (A) and in vivo (B) at 37 °C.

ing the Dixon plot. The time course of α-glucosidase displayed nonlinearity with increasing concentrations of **4** and **5**, i.e., an anomalous time course was obtained as a result of inhibition. This may be attributed to inhibitor binding to the enzyme. The control measured K_m values of α-glucosidase towards maltose and β-glucuronidase towards *p*-nitrophenyl β-D-glucuronide are 2.5 ± 0.3 and 2.3 ± 0.2 mM, respectively. The K_m of α-glucosidase agrees closely with that reported previously [30].

Recently, the toxicity of ethanolamines from single and repeated exposures, including their potential to cause mutation, birth defects and tumors, was reported [31]. Therefore, the in vivo inhibition of α-glucosidase and β-glucuronidase by **4** and **5** may be due to their effect on some biological processes in the cell, such as the inhibition of the tested enzyme synthesis or by their interference with enzyme regulation [5,20,21]. The high affinities of **4** and **5** on the tested glucosidases, due to the ionic polar site, especially β-glucuronidase, can be deduced. The affinity of various amines on the anionic site of enzymes has been reported in the case of almond β-glucuronidase [32,33].

In order to examine whether protonation or deprotonation of ethanolamines is important in the inhibition of glycosidases, the pH dependence of in vitro treated enzymes was investigated. The pattern of the effect of pH on the relative activity of each enzyme is the same in the absence and presence of **4** or **5** (Fig. 5). pH optima are unchanged where pH values of 4.5 and 4.0 are shown for α-glucosidase and β-glucuronidase, respectively.

Inhibition of sweet almond β-glucosidase.— The inhibitory activity of phenyl 6-deoxy-6-(morpholin-4-yl)-α-D-glucopyranoside (**6**) on the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside (ONPG) by β-D-glucosidase from sweet almond was determined under the following conditions: total reaction volume, 1050 μL, pH 6.8 (0.07 M potassium dihydrogen phosphate and disodium hydrogenphosphate buffer); temperature 30 °C; enzyme concentration 0.2 mg/mL; stock solution of inhibitor 1.799 mM at pH 6.8; and substrate concentrations of 20.00, 10.00, 5.00, 3.33, 2.50 and 2.00 mM. The assay method was based on measuring the continuous release of *o*-nitrophenol from *o*-nitrophenyl β-D-glucopyranoside by the action of the enzyme. The K_m at pH 6.8 was determined to be 6.9 mM. The K_i value of compound **6** was determined to be 1.0×10^{-5} M, and the inhibition was found to be competitive.

In conclusion, the data of the present study show that **6** exerts a relatively potent inhibition on α-glucosidase ($K_i < 1.6 \times 10^{-5}$ M), whereas **4** and **5** show less inhibition (Fig. 4). Compound **6** gave a noncompetitive pattern of inhibition of this enzyme, while **4** and **5** showed mixed and competitive types of inhibition, respectively. On the other hand, β-glucuronidase was not inhibited by **6**, but was inhibited by **5** ($K_i < 5 \times 10^{-5}$ M) in a competitive manner. The β-glucosidase from sweet almond was found to be inhibited competitively by **6**, which is more potent than **3** ($K_i < 1.3 \times 10^{-4}$ M) [27]. This may be due to the difference of the anomeric configuration that can possibly play a role on the type of inhibition whereby the β-linkage in **6** makes

the sugar part available in a competitive manner with respect to the substrate. On the other hand, derivatives of **5** were found to act on yeast α-glucosidase in a competitive manner [34], whereas these compounds act in an uncompetitive manner on sweet almond β-glucosidase [35]. Therefore, completely different inhibition mechanisms seem to be operative. Further studies with other enzymes and inhibition analyses are needed in order to clarify the structural requirements in the skeleton of the inhibitor.

3. Experimental

Chemicals and reagents.—*p*-Nitrophenol, *p*-nitrophenyl β-D-glucuronide and bovine

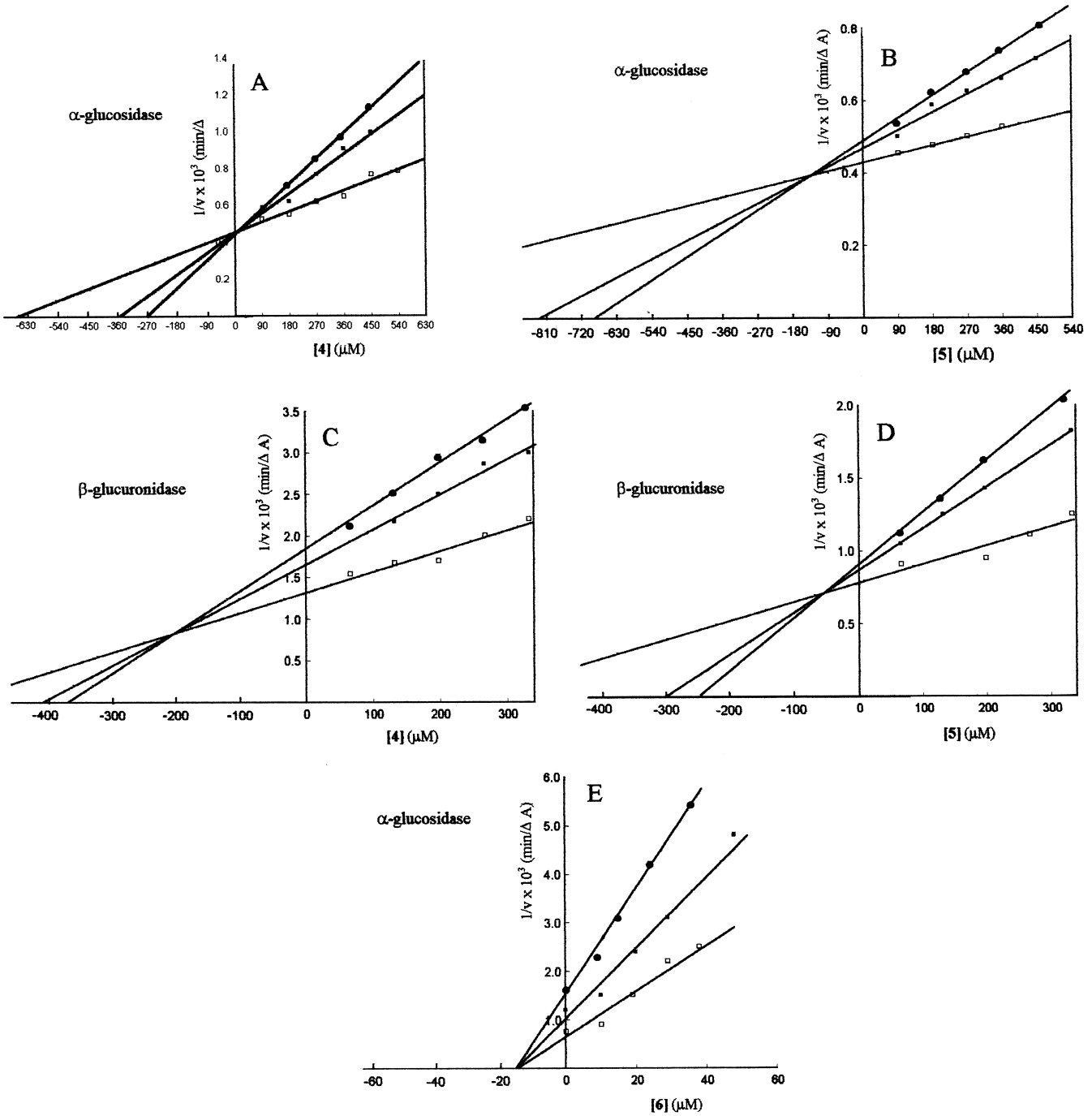


Fig. 4. Dixon plot of in vitro inhibited enzymes. For α-glucosidase: varying concentrations of **4** and **5** and **6** at 7 (●), 14 (■) and 28 (□) μM maltose at 37 °C and pH 4.5, v_0 is expressed as ΔA_{500}/min. For β-glucuronidase: varying concentrations of **4** and **5** at 0.5 (●), 2 (■) and 8 (□) μM *p*-nitrophenyl β-D-glucuronide at 37 °C and pH 4.0, v_0 is expressed as ΔA_{420}/min.

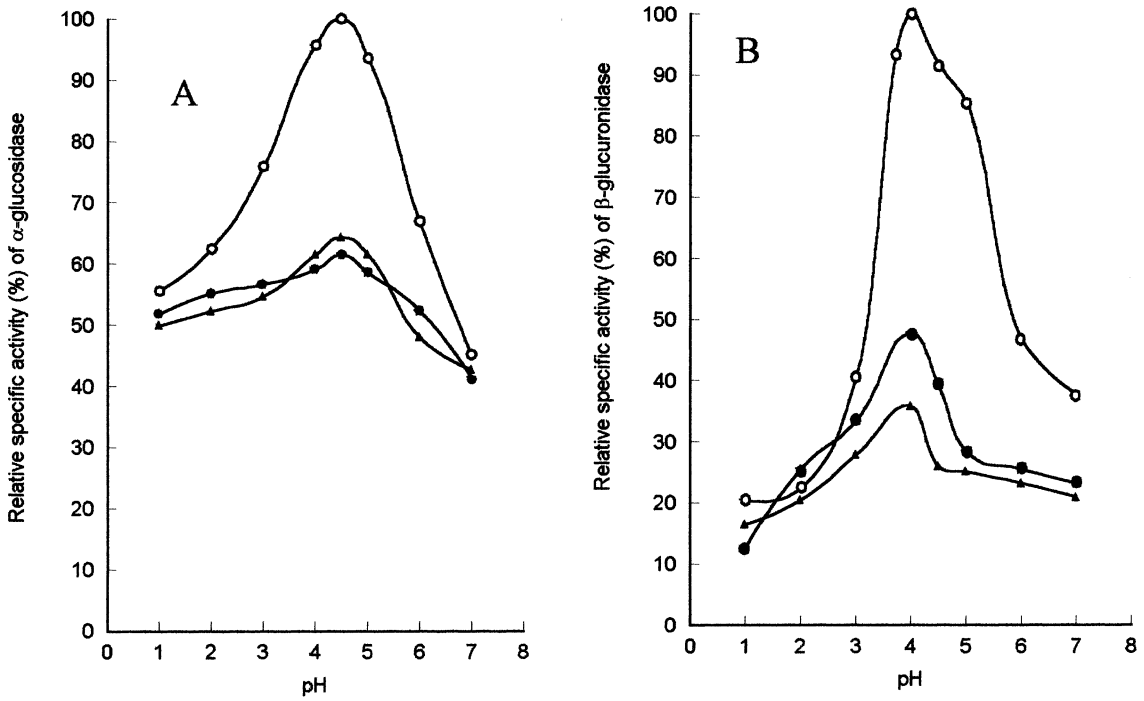


Fig. 5. pH dependence of α-glucosidase and β-glucuronidase in absence (○) and presence of 0.4 mM **4** (●) or **5** (▲).

serum albumin were purchased from Sigma (MO, USA); β-D-glucosidase (sweet almond) and *o*-nitrophenyl β-glucopyranoside (ONPG) were obtained from Boehringer, Mannheim. DEAE-cellulose (DE-52) was from Whatman; Sephadex G-100 was from Pharmacia; the enzymatic colorimetric assay kit for glucose was from Diamond. Other reagents were of analytical grade.

General methods.—Melting points are uncorrected. Optical rotations were measured at 20 °C with a Perkin–Elmer 241 MC polarimeter. The NMR spectra were recorded with Bruker AC 250 and 200 MHz spectrometers. Chemical shifts δ were reported in ppm relative to TMS as internal standard, and described as: s (singlet), d (doublet), t (triplet), m (multiplet) or brs (broad singlet). Column chromatography was performed under normal pressure with E. Merck Silica Gel, 70–230 mesh ASTM and 230–400 mesh ATSM for flash chromatography. Mass spectra were recorded using electron ionization (EI) on a Varian MAT 311A spectrometer and fast-atom bombardment (FAB) on a Kratos MS 50 spectrometer. UV data were recorded with a Philips PU 8740 UV–vis spectrometer. IR spectra were recorded with a Unicam SP 1025 spectrometer. Microanalyses were performed in the Microanalysis Unit at Alexandria University.

*Phenyl 6-deoxy-6-(morpholin-4-yl)-β-*D*-glucopyranoside* (**6**).—A solution of **7** (1 g, 2.43 mmol) in dry DMF (1 mL) was treated with morpholine (0.42 g, 4.82 mmol). The reaction mixture was heated on a water bath for 12 h. The resulting solution was evaporated to dryness under reduced pressure. The resulting syrup was purified by column chromatography using ethyl acetate as eluent to give **6** as white crystals (0.35 g, 44% yield); mp 177–179 °C; $[\alpha]$ +43° (*c* 1, $CHCl_3$); IR: 3340 cm^{-1} (OH).

*Phenyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-(morpholin-4-yl)-β-*D*-glucopyranoside* (**9**).—An ice-cold solution of **6** (0.1 g) in dry pyridine (5 mL) was treated with acetic anhydride (5 mL). The reaction mixture was left overnight at room temperature, then poured on ice-cold water with stirring. The product that formed was filtered, washed with water and crystallized from EtOH to give **9** as white crystals

(0.13 g, 56% yield): mp 184–186 °C; IR: 1751 cm^{-1} (OAc); ^1H NMR ($CDCl_3$, 600 MHz): δ 2.03, 2.04, 2.05 (3s, 9 H, 3Ac), 2.49 (m, 4 H, CH_2-N-CH_2), 2.53 (m, 2 H, H-6,6′), 3.64 (t, 4 H, CH_2-O-CH_2), 3.77 (m, 1 H, H-5), 5.02 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 5.09 (t, 1 H, $J_{3,4}$ 9.33, $J_{4,5}$ 9.47 Hz H-4), 5.21–5.27 (m, 2 H, H-2 and H-3) and 6.99, 7.06, 7.28 (d, t and m, 5 H, ArH); ^{13}C NMR (CD Cl_3): δ_c 54.64 ($N-C_2$), 58.86 (C-6), 67.05 ($O-C_2$), 70.33 (C-4), 71.34 (C-2), 73.00 (C-3), 73.32 (C-5), 99.09 (C-1-β), 117.09, 123.28, 129.53 (C_6H_5), 169.36, 170.31 (C=O) and at 20.64, 20.78 (Ac). Anal. Calcd for $C_{22}H_{29}NO_9$: C, 58.52; H, 6.47; N, 3.10. Found: C, 59.28; H 6.93; N, 3.04.

Animals and in vivo treatments.—Male Swiss albino mice, weighing about 20 g, were housed four per cage (60 × 30 × 20 cm) and had free access to food and water. They were kept under conventional conditions of temperature, humidity and photoperiod for 12 h. Compounds **4** and **5** were prepared as 5 M amine–HCl buffer, pH 7.0 from which different doses (5–50 mg) were prepared for the treatment groups in 0.9% saline base (five mice were used per group for each dose). Mice received intraperitoneally **4**, **5** or **6** in a total volume of 500 μL. Each group was treated for only 24 h and control groups received vehicle only.

Enzyme purification.—α-Glucosidase was purified from mouse liver with 7.0% recovery and purification of 1200-fold, which is near to that described previously. The highest activity was 5279 mU/mg of protein. β-Glucuronidase purified from mouse liver had 9.4% recovery and a 43-fold purification.

The purification of α-glucosidase from normal mouse liver was done as described previously [5]. Livers were homogenized in 9 vols of a cold solution of 1 mM EDTA, pH 7.2 containing 0.25 M sucrose using a Potter–Elvehjem Teflon homogenizer. The homogenate was adjusted to pH 5.0 by 1 M HCl and was then centrifuged at 2,000 rpm for 30 min. The supernatant was discarded, and the pellet was washed, resuspended and homogenized in 1 mM sodium acetate buffer, pH 5.0 containing 0.25 M sucrose. The enzyme was fractionated in the supernatant by 60% ammonium sulfate, the pellet was resuspended in 1 mM EDTA, pH 5.0 containing 25 mM NaCl, and the supernatant was dialyzed against the same buffer. Finally, the dialyzate was applied to a Sephadex G-100 column that was equilibrated with the same buffer.

Purification of β-glucuronidase from mouse liver was performed as reported [36] with some modifications. Livers were homogenized with 9 vol of 5 mM Tris–HCl buffer, pH 7.8 containing 0.25 M sucrose by a Potter–Elvehjem Teflon homogenizer, followed by centrifugation at 3,000 rpm for 10 min. The supernatant was centrifuged at 17,000 rpm for 20 min. The pellet was then suspended in the same buffer and homogenized. The extract was dialyzed against 5 mM Tris–HCl buffer, pH 7.8. The dialyzate was adjusted to 30% ammonium sulfate and centrifuged at 3,000 rpm. The supernatant was adjusted to pH 6.0 and was then brought to a final concentration of 70% ammonium sulfate. After centrifugation, the pellet was dissolved in 5 mM Tris–HCl buffer, pH 7.8, dialyzed and applied to a DE-52 column.

Enzyme assays.—Assay of α-glucosidase is based on incubation with maltose followed by the assay of liberated glucose with glucose oxidase [37]. The assay mixture contained 28 mM maltose and 50 mM maleate at pH 4.5. The reaction was run by the appropriate amount of the enzyme at 37 °C for 60 min in a final volume of 1.1 mL. The liberated glucose was determined as described by Trinder [38] at 500 nm. One unit of enzyme activity hydrolyzes 1 μmol of the disaccharide per min at 37 °C.

The β-glucuronidase activity was assayed using 1 mM *p*-nitrophenyl β-D-glucuronide as a substrate in 0.1 M sodium acetate buffer (pH 4.0). Amounts of the liberated *p*-nitrophenol were measured at 420 nm. One unit of β-glucuronidase activity represents the activity that liberates 1 μmol of *p*-nitrophenol per min at 37 °C [39].

In vitro treatment of enzymes.—Purified enzymes from untreated mice were incubated with different amounts of **4** (0–30 μg), **5** (0–50 μg) or **6** (0–16.25 μg) at 37 °C for 5 min before being assayed for enzymatic activities as described above. The optimum pH for each enzyme was adjusted for **4** and **5** by 1 M HCl before use to be 0.01 M amine–HCl buffer.

Kinetic study of α-glucosidase and β-glucuronidase.—The time course of the reaction of each enzyme and its substrate was determined at pH 4.5 and 4.0 for α-glucosidase and β-glucuronidase, respectively, in the absence and presence of added 0.01 M ethanolamine–HCl buffer. The final concentrations of **4** and **5** ranged from 0.09 to 0.4 mM. Also, the time course of α-glucosidase was measured at 9–45 μM of **6**. K_m values were determined by a Lineweaver–Burk plot, and the K_i value was determined through a Dixon plot. The pH dependence of the enzyme activity was determined in the absence and presence of 0.4 mM **4** or **5**. The substrate was dissolved in buffer of the required pH. The buffers used were in the range of pH 1–7.

β-D-Glucosidase (sweet almond) was kinetically assayed in an assay mixture containing 500 μL of 70 mM potassium phosphate buffer, pH 6.8, 500 μL of 42 mM ONPG, and 50 μL of enzyme solution (0.2 mg/mL) at 30 °C [29]. Liberation of ONPG was monitored using PU 8740 UV–vis spectrophotometer at 405 nm, and the initial hydrolysis rate was calculated.

Protein assay.—Protein was assayed as described previously using bovine serum albumin as standard [40].

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