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Comparative evaluation of D-glucosyl thiouronium, glucosylthio heterocycles, Daonil, and insulin as inhibitors for hepatic glycosidases ☆

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Dedicated to Prof. Hassan S. El Khadem for his achievements on the occasion of his 80th birthday

Abstract—Comparison of the in vivo and in vitro effects of *S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)thiuronium bromide (**1**), 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio-1,3,4-thiadiazolin-5-thione (**2**), and 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-1,3-benzoxazole (**3**), as well as the antidiabetics Daonil and insulin on glycosidase enzymes has been investigated. Compound **1** inhibited both α- and β-glucosidases, but the inhibition was more potent with the β-enzyme. Compound **2** was found to be a weaker inhibitor of these enzymes, while compound **3** showed a slight apparent activation.

Keywords: α-Glucosidase; β-Glucosidase; Glucopyranosylthio; Thiouronium; Thiadiazolinthione; Benzoxazole

1. Introduction

Glycosidase enzymes play various important roles in biological processes,[1–3] and consequently modifying or blocking such processes are important for therapeutic or biotechnological applications.[1–10] The inhibitors of such processes may exert their effects by mimicking the enzyme's normal substrate and/or based on their topological resemblance to the postulated transition state. Various inhibitors have been used for the treatment of diabetics, obesity, and hyperlipoproteinemia, as antivirals, particularly against HIV (the causative agents in AIDS), inhibitors of tumor metastasis, antibacterials, and insect antifeedants.[5–7] The design of glycosidase inhibitors with a high degree of specificity and potency, thus ideal inhibitors, has attracted much attention during the last few years, and there is still a need for design and exploration of new lead inhibitors.[1–25]

A number of thio sugar derivatives, thioglycosides, thio-linked oligosaccharides, glycosylamines, and heterocycles linked to polyols have shown potential value as inhibitors.[5–25] Here we explore glycosidase inhibition by new compounds having in common a glucosylthio moiety attached to carbon linked to nitrogen either in an open chain (isourea) or cyclic form (heterocyclic ring). The present study evaluates, *S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)thiuronium bromide (**1**),[26] 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-1,3,4-thiadiazolin-5-thione (**2**),[27] and 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-1,3-benzoxazole (**3**)[28] (Fig. 1) as glycosidase inhibitors, and compares their effects with those of the clinically used antidiabetics. 1-[4-(2-chloro-2-methoxybenzamido)ethylbenzenesulfonyl]-3-cyclohexyl urea (Daonil)[29] and insulin.

2. Results and discussion

Low concentrations of compounds **1**, **2**, and Daonil had no significant effect in vitro on the activity of α-glucosidase in the liver homogenate of mice but higher

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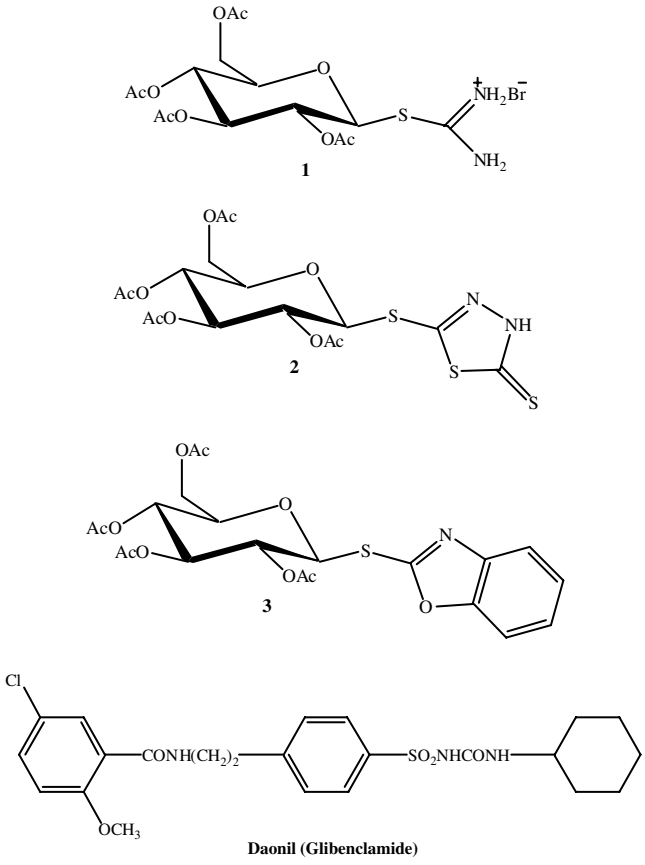

Figure 1. Potential inhibitors.

concentrations of compound **1** showed a mixed type of inhibition. Compound **3** caused no detectable change of the activity of the enzyme (Table 1).

Glycosidase enzymes can be separated from the rat liver homogenate into subcellular fractions containing different α-glucosidases, which are localized in the mitochondrial, lysosomal, microsomal, and cytosolic fractions.[30] The kinetics of inhibition of the lysosomal α-glucosidase fraction, in vitro, by compounds **1** and **3** showed no detectable change of the enzyme activity. Compound **2** caused 23.2% competitive inhibition ($P < 0.05$) and the K_i value was 9×10^{-3} M, as determined from the Lineweaver–Burk plot (Fig. 2). Daonil and insulin showed no significant inhibition ($P > 0.4$). Compounds **1**–**3** and insulin showed no effect in vitro on the microsomal enzyme fraction, whereas Daonil showed slight noncompetitive inhibition of the activity, with 22.3% inhibition ($P < 0.05$) and K_i 12×10^{-3} M (Fig. 3). Compound **1** showed slight competitive inhibition (~24%) of the activity of the cytosolic enzyme, with K_i 7.5×10^{-3} M, v_{max} 5.6×10^{-2} and K_m ranging from 0.20 to 0.32 mM (Fig. 4).

The in vivo activity of the α-glucosidase, eluted from the mitochondrial fraction was inhibited by about 57% and 14% on treating the animals by compound **1** and Daonil, respectively. The higher inhibition of **1** may be attributed to its salt nature, which causes its higher absorption. The relative specific inhibitions of the cytosolic enzyme was 40% by **1** and 47% by Daonil.

The molecular mass of the purified α-glucosidase was estimated by SDS-polyacrylamide gel electrophoresis, staining with Coomassie Blue (Fig. 5). Lane 1 indicated

Table 1. The values of K_m and v_{max} of α-glucosidase activities in liver homogenate under the effect of the compounds studied[a]

Treatment	Kinetic parameters			Probability
	Concentration (μM)	v_{max} (μM/min) 10^{-3}	K_m (mM)	(P)
	Control	4.30	0.14	—
	—			
1	2.60	4.30	0.15	>0.1
	10.30	4.30	0.20	>0.1
	25.70	5.90	0.29	<0.001
	51.30	5.90	0.45	<0.001
2	2.75	4.00	0.14	>0.1
	11.00	3.50	0.14	>0.1
	27.50	3.10	0.14	>0.1
3	2.70	4.76	0.13	>0.1
	10.90	4.65	0.14	>0.1
	27.20	6.89	0.17	>0.4
Daonil	2.50	3.70	0.14	>0.1
	7.50	3.57	0.14	>0.4
	10.00	3.30	0.14	>0.1

[a]The experimental error was determined statistically, from which the probability (P) values were determined: nonsignificant ($P < 0.100$–0.049) and significant ($P < 0.050$–0.001).

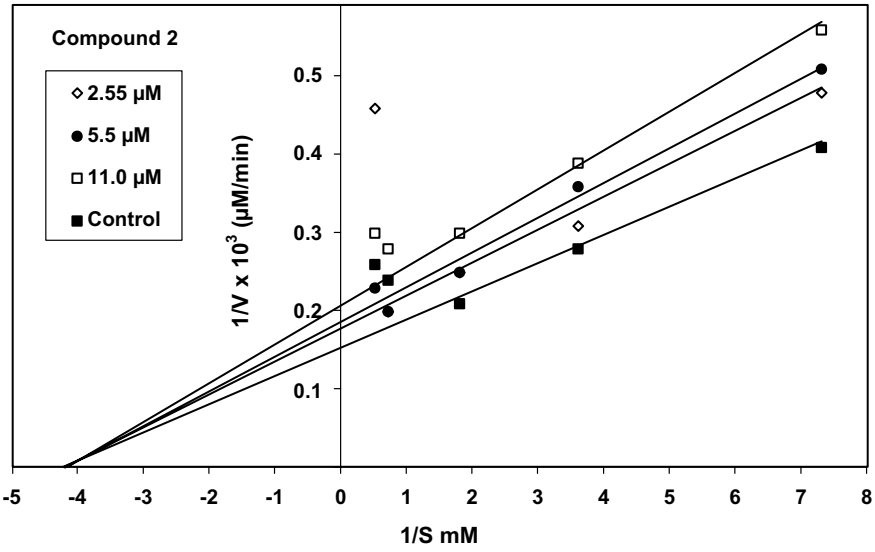

Figure 2. Lineweaver–Burk plot of the inhibition of purified lysosomal fraction of α-glucosidase by compound **2**.

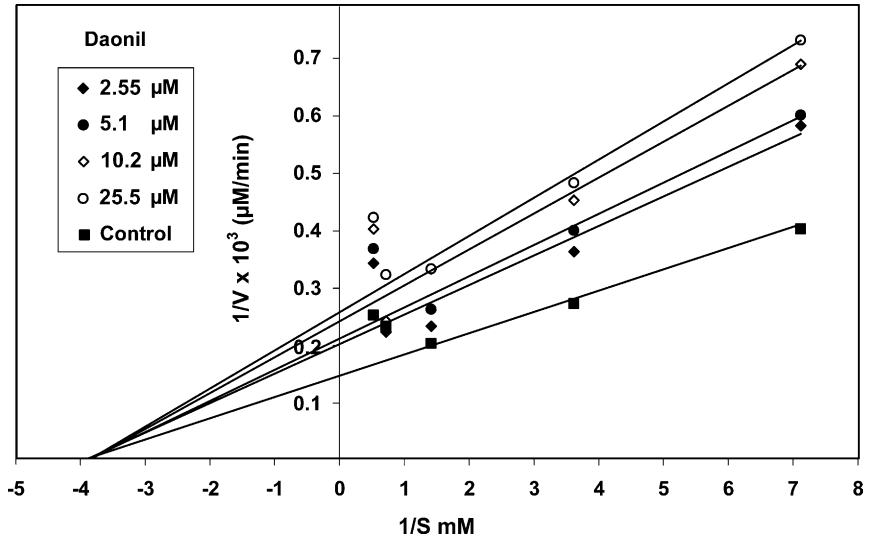

Figure 3. Lineweaver–Burk plot of the inhibition of microsomal fraction of purified α-glucosidase by Daonil.

that the control cytosolic enzyme has three bands, with masses 110,000, 88,000, and 70,000 Da. When the enzyme was treated with compound **1**, it showed only the two bands with masses 110,000 and 70,000 (lane 2). The microsomal fraction had three bands (lane 3) and in the presence of **1** similar bands were obtained (lane 4). On the other hand, there was no clear difference in the electrophoretic patterns of the lysosomal fraction (lane 5) and that treated by **1** (lane 6). Lane 7 shows the electrophoresis of standard proteins, γ-globulin, albumin, and polysterin, with molecular weights of 150,000, 70,000 and 50,000 Da, respectively. The electrophoretic results and the scanning of the in vivo inhibition of the different fractions of α-glucosidase were found to be in essential accordance with the biochemical results.

The study was also extended to β-glucosidases.[30–35] Chromatographic purification of β-glucosidase under controlled conditions gave a well-separated main peak. When treated by compound **1** or **2**, this main peak was accompanied by a second maximum peak. The activity of β-glucosidase was affected by compound **1** to a greater extent than that of α-glucosidase.

Using the *p*-nitrophenyl β-D-glucopyranoside as substrate, compound **1** inhibited the liver homogenate β-glucosidase in vitro with apparent v_{max} 0.20 µM/min and K_m 5.30 mM (Table 2). Compound **2** was found to act as a competitive inhibitor, with v_{max} 0.28 µM/min and K_m 7.10 mM (Table 2). A Lineweaver–Burk plot[36] showed that compound **1**, in low concentrations (5.3–10.4 µM), competitively inhibited the activity of the

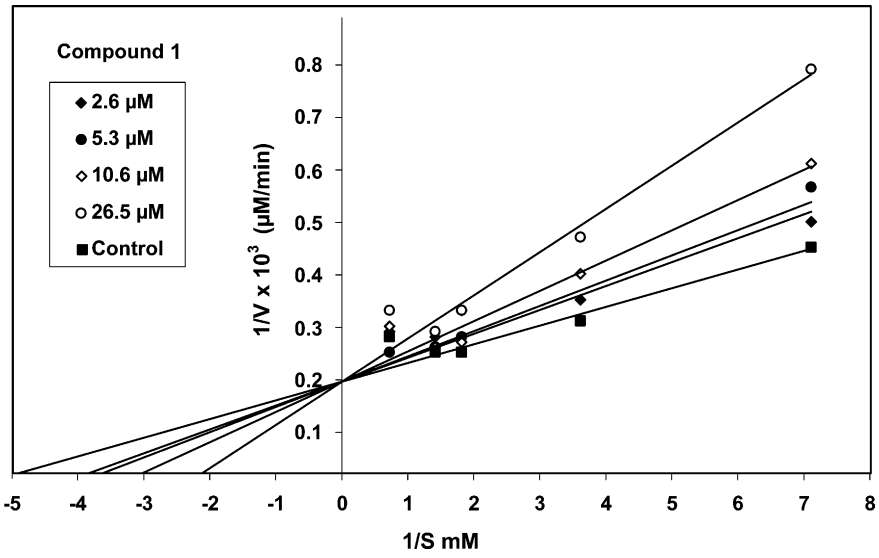

Figure 4. Lineweaver–Burk plot of the inhibition of purified cytosolic fraction of α-glucosidase by compound **1**.

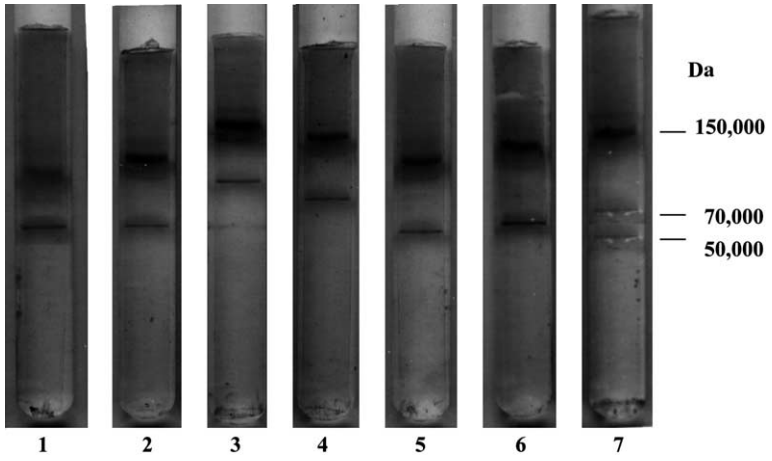

Figure 5. Electrophoretic behavior of purified fractions of α-glucosidase on polyacrylamide gel. Lanes 1, 3, and 5: cytosolic, microsomal, and lysosomal fractions of purified α-glucosidase from liver of control mice; Lanes 2, 4, and 6: represent cytosolic microsomal and lysosomal fractions of purified α-glucosidase from treated mice with effective dose of compound 1; Lane 7: standard of γ globulin, albumin and polysterin.

Table 2. The values of K_m and v_{max} of β-glucosidase activities using *p*-nitrophenyl β-D-glucopyranoside as substrate

Treatment	Kinetic parameters	
	K_m (mM)	v_{max} (µM/min)
Control	4.30	0.28
Compound **1**	5.30	0.20
Compound **2**	7.10	0.28
Compound **3**	4.30	0.39
Daonil	4.30	0.37

purified β-glucosidase (up to 85.6%) with a high significant value ($P < 0.001$), while in high concentrations (15.6–20.8 µM) it showed a mixed type of inhibition of activity, which reached 14.4% (Fig. 6) in a manner similar to that reported for amylases.[37–39] The relative percent inhibition of β-glucosidase in vivo by compounds **1** and **2** were ~70% and 60%, respectively, while Daonil and insulin caused no significant effect, and compound **3** caused rather slight activation (Table 3).

Gel electrophoresis showed that the control β-glucosidase from control mice has four bands; a main one with a mass of 240,000 Da and faint bands with masses 200,000 and 60,000 plus a fourth one with mass less than 50,000 Da (Fig. 7, lane 1). The enzyme treated with compound **1** gave electrophoretic bands corresponding mainly to about 60,000, 66,000, and a broad band between 180,000 and 200,000 Da. Compound **1** seems to separate the main band of the control β-glucosidase with mass 240,000 Da into subunits, each having molecular weight of about 60,000 Da. The scanned electrophoretic pattern of the activity of β-glucosidase under the effect of compound **3**, Daonil, and insulin was not different from that of the control enzyme (Fig. 7). On the other hand, under the effect of compound **2**, the main band

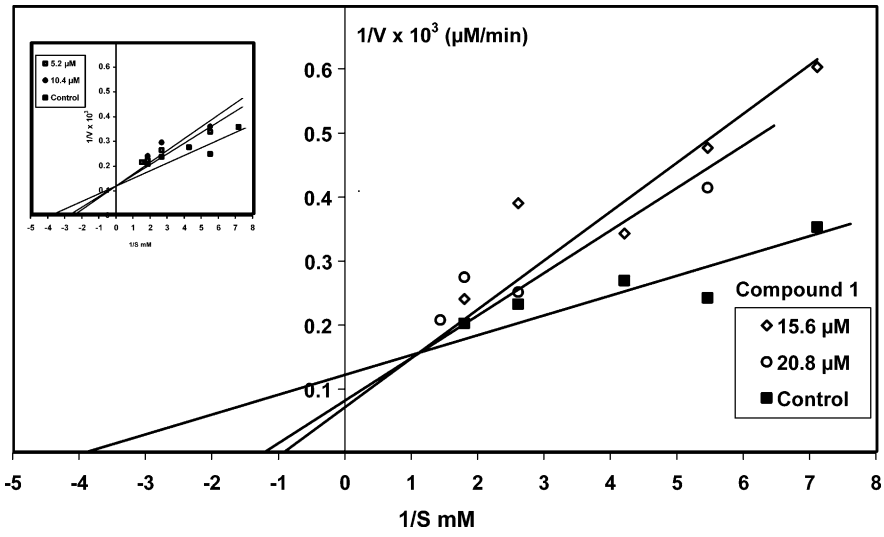

Figure 6. Lineweaver–Burk plot of the inhibition of purified β-glucosidase by high concentrations of compound **1** using maltose as a substrate; the upper drawing by using a small concentrations.

Table 3. The relative inhibition and relative specific inhibition of β-glucosidase using *p*-nitrophenyl β-D-glucopyranoside as specific substrate

Compound	Relative inhibition (%)	Relative specific inhibition (%)
1	48.0	69.5
2	27.0	60.0
3	Slight activation	Slight activation
Daonil	18.9	Nonsignificant
Insulin	15.0	Nonsignificant

present in the control β-glucosidase, was missing from the scanned picture, while a faint band appeared with molecular mass higher than 240,000 Da. Compound **2** in vivo inhibited the purified β-glucosidase by ~27% (Fig. 7 and Table 3).

The types of inhibition of α- and β-glucosidases in vitro by compound **1** were similar, being mixed inhibitors at higher concentrations and competitive ones with lower concentrations. Compound **1** in vitro caused competitive inhibition of the purified cytosolic α-glucosidase (Fig. 4). This may be due to its higher affinity for the cytosolic enzyme, and/or that the cytosolic enzyme is in a more soluble state than the other fractionated α-glucosidases. Kinetic evidence for two active sites has been reported for glucosidases; active site 1 has more

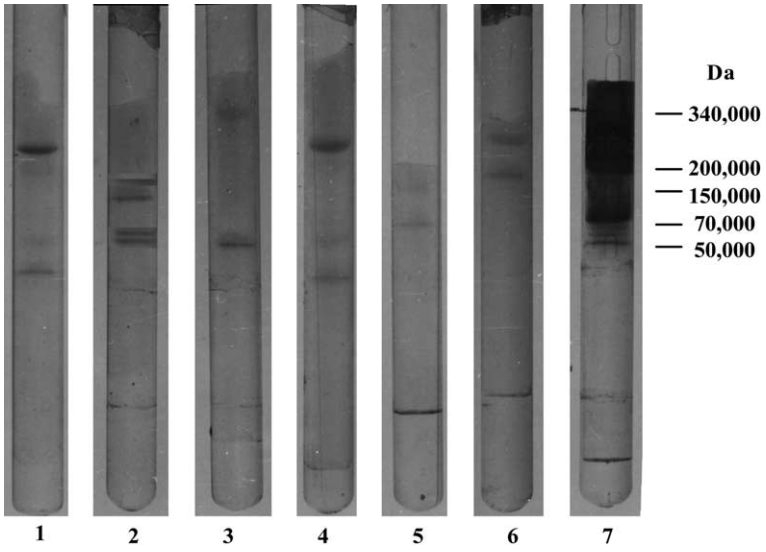

Figure 7. Electophoretic behavior of purified β-glucosidase on polyacrylamide gel. Lane 1: β-glucosidase of control mice; Lane 2: β-glucosidase of mice treated with compound **1**; Lane 3: β-glucosidase of mice treated with compound **2**; Lane 4: β-glucosidase of mice treated with compound **3**; Lane 5: β-glucosidase of mice treated with Daonil; Lane 6: β-glucosidase of mice treated with insulin; Lane 7: Standards proteins (fibrinogen, α1-lipoprotein, γ-globulin, albumin, polysterin).

affinity than site 2 for synthetic substrates.[40,41] Maltose as substrate could bind with site 2 of β-glucosidase by one of its glucose moieties, whereas the other moiety would be located close to site 1, hindering but not excluding the binding of the substrate (PNP-Glc) to this site. In this case compound **1** may find its way to site 1 of the enzyme, thus competing with the substrate and causing enzyme inhibition in accord with reported data.[37] The resulting glucose apparently behaves as a pure competitive inhibitor to the glucosidases when compound **1** is used in low concentration (the upper drawing of Fig. 6); thus competing with the substrate in the active site 1. Alternatively maltose may act as a nonspecific substrate towards β-glucosidase in the absence and in the presence of compound **1** and the other studied compounds, taking into consideration that the concentration of the substrate maltose was found to be twice as much as that of the *p*-nitrophenyl β-D-glucopyranoside.[40]

Compound **2** in vitro showed slight competitive inhibition of β-glucosidase (Table 2) when compared to compound **1**. This may be attributed to the presence of the amino group in **1**, which may lead to a better interaction with the enzyme, but less effectively than the functional group in compound **2**. Moreover, compound **3** and Daonil were less effective than **2** and caused no enzyme inhibition.

Comparison of the effect of the compounds studied on blood sugar level was made to determine the relation between their inhibitory actions on the glucosidases and the antihyperglycemic behavior. Compound **1** caused little reduction of blood sugar level. Thus, the blood sugar levels in mice treated with 0.93 and 0.185 mg/g body weight of compound **1** were 90% and 80% relative to control, whereas those mice treated with insulin (0.04, 0.05 and 0.06 IU/animal) was reduced to 58, 55 and 53 mg/dL, respectively, compared to control. Compound **2**, in a low dose, did not cause a change in blood sugar level whereas higher doses (0.74 mg/g body weight) reduced the level to 56.6%, but a complete depression of the animal took place as a side effect. Daonil in small doses (0.07 mg/g body weight) unexpectedly increased the blood sugar level to ~160% relative to control, while higher doses (about 0.74 mg/g body weight) decreased the level to 65% relative to the control. For comparison, few studies have been carried out in diabetic humans, but it was found that Daonil given daily (2–3 tablets) did not reduce the blood sugar level with some patients.

The combination of compound **1** and Daonil appeared to cause some potentiation in lowering the blood sugar level in mice, but there were other toxic effects, leading to complete depression of the animal within 10–15 min. In spite of the fact that insulin decreased the blood sugar level to 50% relative to control with different doses (0.04–0.06 IU/animal), it did not show any effect on the activities of the α-glucosidase nor on the β-glucosidase.

3. Experimental

3.1. Materials

p-Nitrophenyl β-D-glucopyranoside, phenylmethylsulfonyl fluoride, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), and Sephadex G-150 were purchased from Sigma (St Louis, MO, USA). Sephadex G-100 and hydroxyapatite gel were from Pharmacia (Uppsala, Sweden). DE-52 cellulose (pure) was from Whatman. Daonil tablets, each containing 5.0 mg of glibenclamide, were a product of Hoechst Orient SAE (Cairo, RCC). Insulin was provided in 10 mL suspension containing 40 IU/mL. Mixtard 30HM (Biophasic isophane insulin infection 30170). Biosynthetic insulin was from Egyptian Drug Trading Company. Triton X-100, Folin–Ciocalteau phenol reagent, maltose, and other reagents were of analytical grade. Ethylenediaminetetraacetic acid (EDTA) was obtained from British Drug Houses England. Potassium dihydrogenphosphate was purchased from Veb Labor Chemie Apold, Germany. The enzymatic colorimetric assay kit for glucose was purchased from Boehringer, Mannheim, Germany. Electrophoretic reagents used were: acrylamide 30% for running gel (separating), acrylamide 7.9% (spacer gel), running gel buffer, pH 8.9, spacer gel buffer, pH 6.7, stock reservoir buffer 0.5 M tri-glycine, pH 8.3. The destaining solution was 0.1% bromophenol blue in 7% acetic acid and 10% MeOH. Coomassie stain was 0.1% Coomassie Brilliant Blue R 250 in 50% MeOH and 10% acetic acid. Compounds studied were: *S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthiouronium) bromide[26] (**1**), 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-1,3,4-thiadiazolin-5-thione[27] (**2**), and 2-(2,3,4,6-tetra*O*-acetyl-β-D-glucopyranosylthio)-1,3-benzoxazole[28] (**3**).

3.2. Instruments

The spectrophotometer was from Amershman Pharmacia Biotech, wavelength range: 200–900 nm. LKB 2117 Multipho R was used for electrophoresis. Scanning was carried out in an Epson GT-9600 apparatus.

3.3. Animals and in vivo treatments

Male Swiss albino mice were obtained from the animal house of the Medical Research Institute, Alexandria University. The animals were 10 weeks old with an approximate body weight of 27 g. They were housed in

cages for three days under conventional conditions and supplied with a diet consisting principally of whole milk and bread.

Solutions of compounds **1**–**3** and Daonil were prepared in concentrations 37.0, 43.5, 43.0, and 41.0 mM, respectively. Different doses were prepared in 0.9% saline solution. All compounds were administrated by subcutaneous injection except for Daonil, which was administrated orally. Doses of compounds **1**–**3** and Daonil were 0.66–1.48, 0.74–1.59, 0.74–1.59, and 0.4–1.48 mg/g body weight, respectively. Insulin was given in doses 0.04–0.08 IU/animal according to body weight by using a special insulin syringe. Each group of animals contained six mice. The LD_{50} of these compounds was determined by giving different doses of each compound. A control group received vehicle only. The livers were then taken for enzyme purification and assay.

3.4. Purification and fractionation of α-glucosidase

At the end of each period of treatment, mice were sacrificed and cardiac puncture blood was collected in vials without anticoagulants. The serum was assayed for blood sugar, and the tissue was then obtained by dissection. Enzyme purifications were carried out according to the known methods.[42,43]

3.5. Purification and isolation of β-glucosidase

Fifty mice treated with compounds **1**–**3**, Daonil, and insulin and 10 normally fed mice were killed by diethyl ether. The livers were removed and homogenized in seven volumes of 10 mM sodium phosphate buffer, pH 7.0. Electrophoretic studies for both α- and β-glucosidases were carried out using sodium dodeocyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to literature methods.[44]

3.6. Enzyme assays

Assay of α-glucosidase was based on its incubation with maltose as a substrate followed by assay of the liberated glucose with glucose oxidase.[40] The assay mixture contained 0.1 mL of enzyme. 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 in 50 mM maleate buffer, pH 4.5. The liberated glucose was estimated as reported earlier. β-Glucosidase assay[40] employed incubation with 0.1 M *p*-nitrophenyl β-D-glucopyranoside as substrate in a 0.2 M sodium acetate buffer, pH 5.5 in a final volume of 0.5 mL. The reaction was then stopped by the addition of 500 μL of 0.2 M sodium carbonate–hydrogen carbonate buffer, pH 10.4, followed by determination of the liberated *p*-nitrophenol, which forms a yellow chromophore in alkaline media with maximum absorbance at 410 nm at 37 °C.

3.7. In vitro treatment of enzymes

The appropriate amounts of homogenate and purified enzyme fractions from untreated mice were preincubated with concentration of 2.60–51.30, 2.75–27.50, 2.70–27.20, 2.50–25.50 μM of compounds **1**–**3** and Daonil, respectively, as well as 0.02–0.04 IU/mL of insulin. Liver homogenates from untreated mice were preincubated with concentrations of 29, 22, 22, and 20.3 μM of compounds **1**–**3** and Daonil, respectively. Incubation was carried out with the homogenate and the purified β-glucosidase preincubated with 5.2–20.8 μM of compound **1**. The activities of homogenate and purified fractions of α-glucosidase and of the purified β-glucosidase were determined at pH 4.5 for α-glucosidase in the absence and presence of different concentrations of studied compounds, whereas the reaction of β-glucosidase was determined at pH 5.5. K_m values were determined by Lineweaver–Burk plots and K_i was determined from the plot of kinetic data. Protein contents were determined as described in literature.[45] The statistical analysis used the *t*-value test.

3.8. Determination of blood sugar level

After treatment of mice with different doses of studied compounds, the mice were sacrificed, blood was collected, and the blood sugar level was determined, as reported in the literature.[46]

Acknowledgements

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