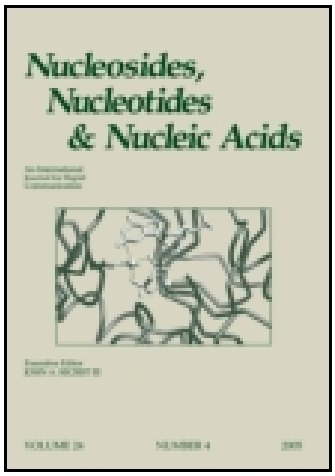

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn20

INHIBITION OF SOME HEPATIC GLYCOSIDASES BY THE DISECO NUCLEOSIDE, 4-AMINO-3-(D-GLUCOPENTITOL-1-YL)- 5-MERCAPTO-1,2,4-TRIAZOLE AND ITS 3-METHYL ANALOG

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Published online: 17 Aug 2006.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 21, No. 10, pp. 695–708, 2002

INHIBITION OF SOME HEPATIC GLYCOSIDASES BY THE DISECO NUCLEOSIDE, 4-AMINO-3-(D-GLUCOPENTITOL-1-YL)-5-MERCAPTO-1,2,4-TRIAZOLE AND ITS 3-METHYL ANALOG

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ABSTRACT

The in vivo and in vitro effects of 4-amino-3-(D-glucopentitol-l-yl)-5-mercapto-1,2,4-triazole and its 3-methyl analogue on α- and β-glucosidases, β-glucuronidase as well as α-amylase have been investigated. α-Glucosidase is the enzyme that is markedly affected in vivo and in vitro in a dose-dependent manner. The compounds showed a reversible inhibition of a competitive type for α-glucosidase. Moreover, they exert a relatively potent inhibition on α-glucosidase with a K_i magnitude of 3.6×10^{-4}, 9.5×10^{-5} M.

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DOI: 10.1081/NCN-120015726 1525-7770 (Print); 1532-2335 (Online)

INTRODUCTION

Glycosidases are glycoprotein enzymes that hydrolyze glycosidic bonds and play various important biological processes such as digestion, catabolism of glycoconjugates and the biosynthesis of glycoproteins.[1] There are two groups of these enzymes: exoglycosidases such as α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-glucuronidase (β-D-glucuronide glucurono-hydrolase, EC 3.2.1.31)[2] and the endoglycosidases such as α-amylase (α-D-glucan glucanohydrolase, EC 3.2.1.1).[3,4]

Glycosidase inhibitors are compounds capable of slowing or preventing the enzyme catalysis. Reviews[5–13] on that topic indicated the interest of many investigators[14–24] to find potent and selective inhibitors of potential therapeutic and/or biotechnological relevance.[25] Thus, they have a potential value for treatment of some metabolic disorders such as diabetes mellitus,[26–28] lysosomal storage diseases[29–31] and postprandial glycemic rise. They affect the level of insulin, plasma lipids,[32] and tumor metastasis[33] as well as antiviral.[34–37] They are used as antiobesity drugs, fungiostatic,[38] immune modulators[39] and insect antifeedants.[40–42] Nojirimycin and deoxynojirimycin (**1**) (Fig. 1) are potent inhibitors of glucosidases.[43–47] A good competitive inhibition of sweet almond β-glucosidase has been exhibited by the amidine[48] (**2**) and the amidrazone[48,49] (**3**). The 1,2,4-triazole (**4**) competitively inhibited β-glucosidases from sweet almond and *Caldocellum saccharolyticum*[50] in contrast to respective 1,2,3-triazole and tetrazole analogues.[51,52] These aspects attracted our attention to design an acyclic analogue of **4**, by applying the disconnection at the C-N bond, to offer the respective seco-analogue **5**. This assumption has been encouraged by the previous finding that some acyclic analogues showed inhibition, for example 2-deoxy-2-(1-hydroxyeth-2-yl)amino-glycerol which showed a competitive inhibition of yeast α-glucosidase,[53] but exhibited uncompetitive inhibition of β-glucosidase.[54] Moreover, the presence of a basic group as well as functional groups capable of hydrogen bonding may enhance the binding to the enzyme. Thus, as a continuation of our work on the synthesis and evaluation of glycosidase inhibitors[5–7, 54–57] as well as the synthesis of acyclic nucleosides, the in vitro and in vivo effects of 4-amino-3-(D-glucopentitol-l-yl)-5-mercapto-1,2,4-triazole[58,59] (**6**, GT) and its simple analogue 4-amino-3-methyl-1,2,4-triazole[60] (**7**, GM) on purified hepatic α- and β-glucosidases, β-glucuronidase and α-amylase have been studied.

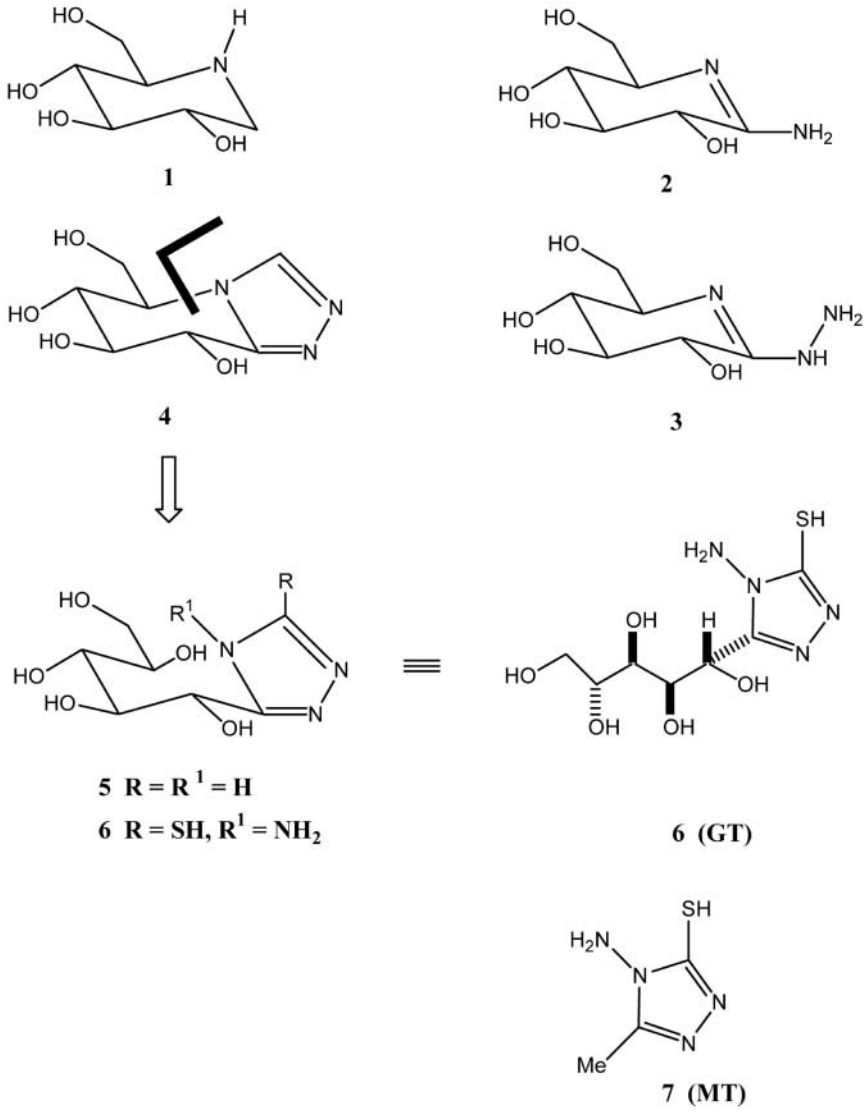

Figure 1.

MATERIALS AND METHODS

Materials

p-Nitrophenol, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside and bovine serum albumin were purchased from Sigma Co. (St louis, Mo, U.S.A) and Sephadex G-100 was from pharmacia (Uppsala, Sweden). MT and GT[58] were prepared as reported earlier. 3,5-Dinitrosalicylic acid (DNS), maltose, starch,

Folin-Ciocalteau phenol reagent, sodium dodecyl sulfate (SDS) and other reagents were of analytical grades.

Animals

Swiss albino mice were obtained from the animal house of the Medical Research Institute, Alexandria University. The animals were 8 weeks old with an approximate body weight of 20 g and housed in wire cages in-groups of six mice per cage. They were kept healthy under conventional conditions of temperature, humidity and a 12 h photoperiod. Mice were supplied with a diet consisting principally of whole milk and bread. Minerals and vitamins were added from time to time. Also, water was continuously provided.

Animal Treatments

Animal treatments were done as described earlier.[61,62] Solutions of 57.7 mM of GT and MT in hot water were prepared. Both compounds were administered by oral ingestion in a dose of 0.10–0.50 mg/g body weight. Six mice were used per group for each dose. Each group of animals was treated for two days (a dose/24 h) and the control group received vehicle only. At the third day the mice were sacrificed and the livers were excised for the purification and assays of the enzymes.

Determination of LD_{50} of Compounds 1 and 2

A group of mice were given different oral doses of GT or MT as mentioned above. The administration of doses occurred for 24 h and repeated again for the same period. Triplicate treatment was done for each dose. Control mice were given only the same volume of water. The tested compounds were made available at the chosen dose levels to achieve test groups with sufficient mortality rates to permit calculation of the LD_{50}. The mice were individually caged and observed for mortality.

Enzyme Purification

The isolation and purification of α-glucosidase from normal mice livers was carried out as previously described.[63] All steps were carried out between 0–4°C, and the purification process included the isolation of the lysosomal extract, concentration with ammonium sulfate and retardation of Sephadex G-100. SDS-PAGE was then carried out according to the known method[64]

using 10% gel. The gel was stained with Coomassie Brilliant blue and then destained for visualization. Partial purification of β-glucuronidase from mice livers was performed as previously described[65] by ammonium sulfate fractionation.

Enzymes Assays

The assay of α-glucosidase is based on the incubation with *p*-nitrophenyl α-D-glucopyranoside as a substrate, followed by the determination of the liberated *p*-nitrophenol that forms a yellow chromogen under alkaline condition with maximum absorbance at 410 nm.[66] The reaction was done at 37°C for 30 min by using the appropriate amount of enzyme and 20 mM *p*-nitrophenyl α-D-glucopyranoside in 50 mM sodium acetate buffer; pH 4.5 in a final volume of 0.5 mL. The reaction was terminated by the addition of 0.2 M Na_2CO_3- $NaHCO_3$ solution, pH 10.4 (0.5 mL), and the liberated *p*-nitrophenol was determined through a standard curve of *p*-nitrophenol. One unit of α-glucosidase activity is defined as the amount of enzyme, which hydrolyzes 1 μ mole of *p*-nitrophenyl α-D-Glucopyranoside per minute at 37°C. Specific activity is expressed as units per mg enzyme protein.

The β-glucosidase assay[67] was carried out at 30°C for 15 min by using the appropriate amount of enzyme and 25 mM *p*-nitrophenyl β-D-glucoyranoside in a 0.05 M sodium acetate buffer, pH 4.6 in a final volume of 0.5 mL The reaction was stopped by the addition of 700 μL of 0.2 M sodium carbonate. The concentration of liberated *p*-nitrophenol was calculated as described for α-Glucosidase.

β-Glucuronidase activity was assayed by using 40 mM *p*-nitrophenyl β-D-glucuronide, as a substrate in 0.1 M soidum acetate buffer, pH 4.0. The assay mixture contained 8 mM *p*-nitrophenyl β-D-glucuronide, 40 mM sodium acetate buffer (pH 4.0), 100 μL enzyme and 0.4 mL water. The reaction was run at 37°C for 2 h in a final volume of 1.0 mL and stopped by adding 4.0 mL of 0.2 M glycine/SDS, pH 11.7. The amount of the liberated *p*-nitrophenol was measured after 10 min.[68]

α-Amylase activity was measured by the increase in the reducing power of a soluble starch solution.[69] The reaction was carried out by incubating 1 mL of diluted enzyme and 1 mL of 0.02 mM soluble starch in 0.02 M sodium phosphate buffer, pH 6.9, containing 0.0067 M NaCl at 25°C for 3 min. The reaction was interrupted by the addition of 2 mL of 3,5-dinitrosalicylic acid reagent. Finally, the mixture was heated for 5 min in boiling water and cooled in running tap water. After the addition of 20 mL of water, the brown color was measured at 540 nm. A calibration curve of maltose was used to convert the colorimeter readings into mg of maltose.

In Vitro Treatment of the Enzymes and Kinetic Studies

The appropriate amounts of purified enzymes from untreated mice were preincubated with different concentrations of GT and MT (0–100 μM) at 37°C for 5 min and the enzymatic activities were then measured. The time course of reaction of α-glucosidase enzyme and its substrate was determined at pH 4.5 in absence and presence of GT and MT at final concentration ranging from 20 to 100 μM. The values of the inhibition constants (K_i) were determined from the double reciprocal plots.

Protein Determination

Protein contents were determined using bovine serum albumin as a standard.[70]

RESULTS

In Vitro Studies

The in vitro effects of various concentrations of GT and MT on the activities of α-glucosidase, β-glucosidase, β-glucuronidase, and α-amylase were investigated as shown in Table 1. The inhibitions of α-glucosidase by the nucleoside GT and its analogue MT were found to take place in a

Table 1. In Vitro Effect of GT and MT on the Activities of Hepatic Glycosidases

Concentration (μM)		Relative Specific Activity[#]		
GT	MT	α-glucosidase	β-glucuronidase	α-amylase
0	0	100.00	100.00	100
20		45.80	97.47	89
40		16.78	99.57	89
60		3.08	94.41	99
80		2.94	94.40	82
100		1.99	95.76	80
	20	92.66	94.00	95
	40	47.90	99.00	89
	60	17.66	98.00	89
	80	13.29	94.00	86
	100	9.16	90.00	85

[#]Ratios of specific activity of enzymes isolated from mice in presence and absence of GT and MT.

dose-dependent manner. The concentration of the compound resulting in 50% inhibition (IC_{50}) of α-glucosidase was found to be 18.5×10^{-6} M and 39.0×10^{-6} M for GT and MT, respectively. On the other hand, both compounds showed a slight inhibition of α-amylase and did not cause detectable changes on β-glucuronidase and β-glucosidase.

The reversibility of inhibition of α-glucosidase was indicated by preincubation of the enzyme with GT and MT at room temperature for 60 min and then dialyzed at 4°C for 16 h, whereby the enzyme activity was fully recovered. Moreover, increasing the time of incubation to 12 h did not affect the recovery of the activity compared to control.

The kinetics of hepatic α-glucosidase inhibition by GT and MT were done. This involved the purification of α-glucosidase from mice liver to homogeneity. The two bands (76 and 69 KDa) on SDS-polyacrylamide gel electrophoresis correspond to the enzyme (Fig. 2). The enzyme was purified with 1200-fold and 11% recovery. Accordingly, the initial velocities of hepatic α-glucosidase were determined at various concentrations of *p*-nitrophenyl

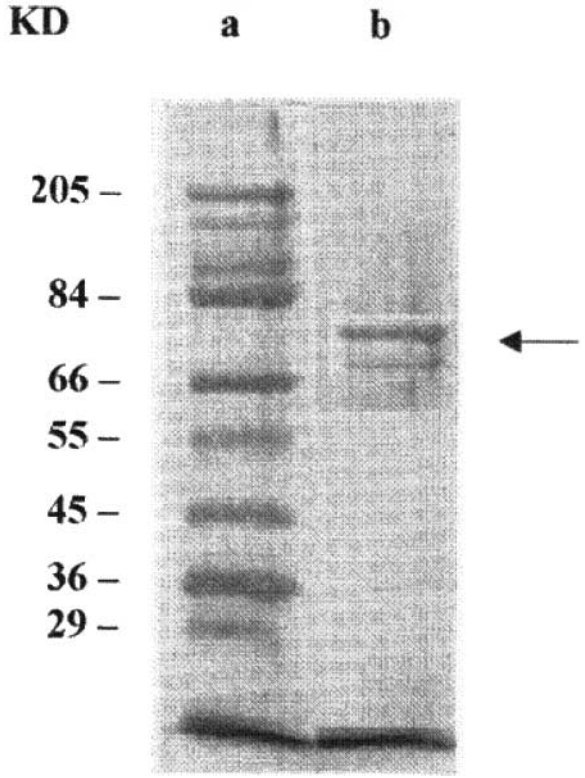

Figure 2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of purified hepatic α-glucosidase. Lane a: Standard markers; lane b: hepatic α-glucosidase subunits.

α-D-glucopyranoside as a substrate in the absence and presence of two fixed concentrations 60 and 100 μM of GT and MT. The Lineweaver-Burk plots of the inhibition pattern are shown in Fig. 3, which indicated that the inhibition by both compounds is competitive with respect to *p*-nitrophenyl α-D-glucopyranoside. The inhibition constant (K_i) values are 3.63×10^{-4} M and 9.46×10^{-5} M for GT and MT, respectively.

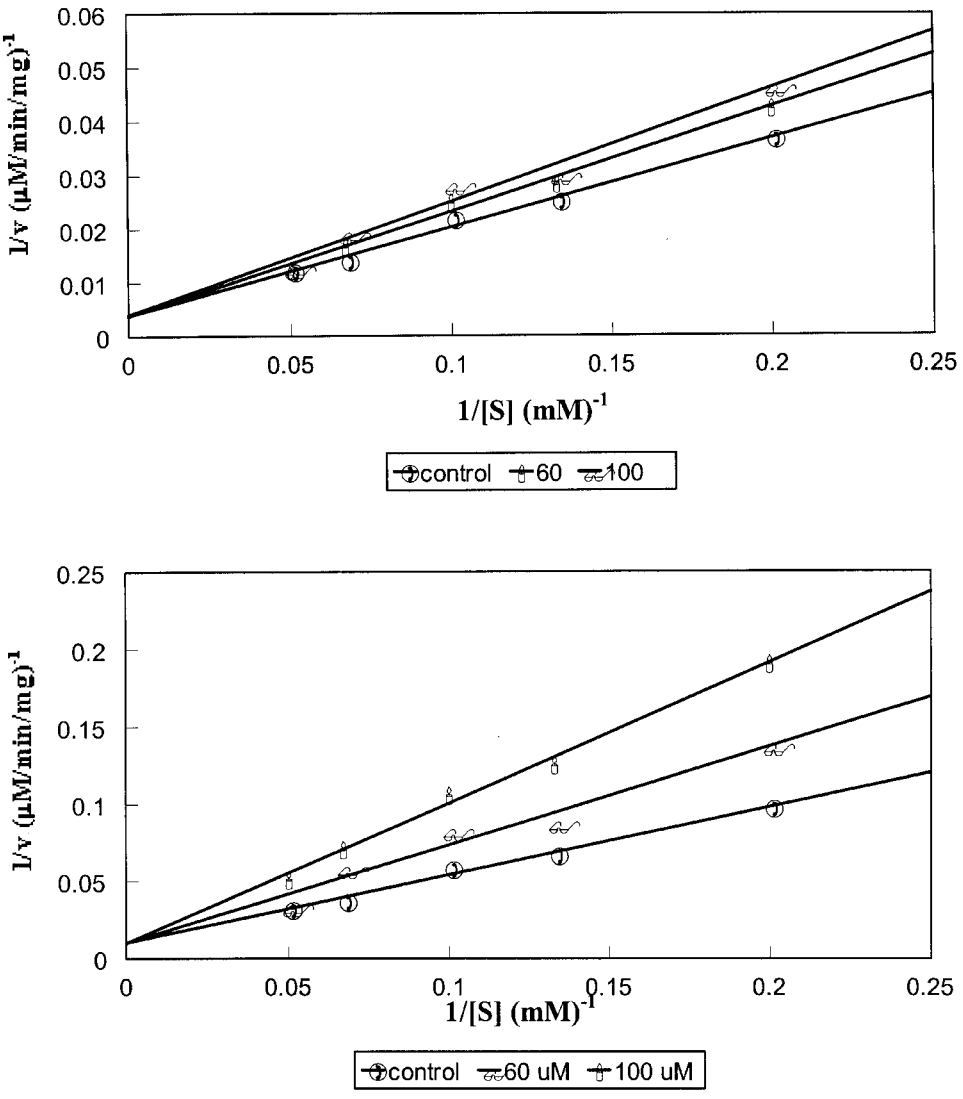

Figure 3. Double reciprocal plots of hepatic α-glucosidase in absence and presence of GT (upper panel) or MT (lower panel).

Table 2. In Vivo Effect of GT and MT on the Activities of Hepatic Glycosidases

Dose (mg/g body wight)		Relative Specific Activity[#]		
GT	MT	α-glucosidase	β-glucuronidase	α-amylase
0	0	100	100	100
0.1		69	65	80
0.2		58	66	77
0.3		50	66	67
0.4		44	68	63
0.5		33	58	63
	0.1	82	81	90
	0.2	80	80	89
	0.3	55	80	85
	0.4	46	77	80
	0.5	37	62	53

[#]Ratios of specific activity of enzymes isolated from mice in presence and absence of GT and MT.

In Vivo Studies

The LD_{50} was found to be 0.4 mg and 0.6 mg/g body weight for MT and GT, respectively. In vivo treatment of mice with GT and MT caused a decrease in the specific activity of hepatic α-glucosidase, β-glucuronidase, and α-amylase in a dose-dependent manner (Table 2), while β-glucosidase was not affected compared to vehicle-treated mice. The hepatic α-glucosidase, β-glucuronidase, and α-amylase, treated with GT, displayed relative activity values of 33.3, 58.0 and 89.0%, respectively. Similarly, they displayed relative activity values of 36.4, 61.6 and 53.0%, respectively on the treatment by MT (Table 2). These data demonstrate that GT and MT showed stronger inhibitory action on α-glucosidase than other glycosidases.

DISCUSSION

The results of the present study showed that in vitro, MT is a somewhat more potent inhibitor of mouse hepatic lysosomal α-glucosidase than GT. On the other hand, the in vitro treatment of β-glucosidase, β-glucuronidase and α-amylase with GT or MT does not show significant changes of their relative specific activities. This result implies that these compounds may selectively or specifically inhibit α-glucosidase. The dose-dependent manner of the in vitro inhibition by GTand MT of the hepatic lysosomal α-glucosidase indicated that a rapid interaction between these inhibitors and enzyme might have taken place, probably occurred at the active site of the enzyme.

The results of dialysis indicated that inhibition is of a reversible type. The ability to recover α-glucosidase activity after treatment with GT and MT rules out a possible covalent interaction with the enzyme. Therefore, we can postulate that both compounds are not tightly bound to the lysosomal α-glucosidase, suggesting that this inhibition could result from their involvement in the formation of an unstable transition state at the active site of the enzyme. Accordingly, the enzyme-inhibitor complex rapidly dissociates and the inhibition would appear reversible. Examples having reversible inhibition of rat hepatic lysosomal α-glucosidase in vitro[17] or slow dissociation from EI complexes of some glycosidases[71–73]or tight binding of α-glucosidase[74] are reported in the literature.

Kinetic studies of the in vitro treated α-glucosidase demonstrate that both GT and MT competitively inhibited the enzyme. This suggests that these inhibitors compete with the nonphysiological substrate for the same active site of α-glucosidase enzyme. The degree of α-glucosidase inhibition by MT ($K_i = 9.46 \times 10^{-5}$ M) is slightly higher than that of inhibition by GT ($K_i = 3.63 \times 10^{-4}$ M). On the other hand, unlike the in vitro inhibition, the in vivo inhibition of α-glucosidase from mice treated with GT and MT were approximately the same at all the given doses. The same potency as inhibitors for hepatic lysosomal α-glucosidase by GT and MT after oral administration may be due to their absorption in the gut and subsequent transport to the liver, where they can come in contact with α-glucosidase and exert their inhibitory effect. In liver cells, some biological processes may be operated in a manner leading to a similar inhibition of lysosomal α-glucosidase by both compounds.

Both GT and MT showed stronger in vivo than in vitro inhibiting activities against β-glucuronidase and α-amylase. This may be attributed to the antagonistic effect, which is exerted by GT and MT for the corresponding physiological substrates acted upon by these glycosidases.

On the other hand, the present data demonstrated that neither GT nor MT showed in vitro or in vivo inhibitory effects on hepatic β-glucosidase. However, it has been reported that nagstatin triazole analogs showed very specific inhibiting activities against β-glycosidases in *E. coli* and snails.[50] Although, β-glucuronidase was not inhibited by phenyl 6-deoxy-6-β-morpolino-B-D-glucopyranoside (PDMG), but inhibited by diethanol amine ($K_i = 5 \times 10^{-5}$ M) in a competitive manner,[57] the latter inhibited β-glucosidase from sweet almond in an uncompetitive type[54] as the acyclic analogues of DNJ.[55] The α-methyl glucoside analogue of PDMG was found to inhibit the β-glucosidase from sweet almond in a competitive manner, but with less magnitude.[55] The anomeric configuration is playing a role on the type of inhibition whereby the β-linkage in PDMG makes the possibility of the sugar part available in a competitive manner with respect to the substrate. Also, galacto- and manno-analogues were demonstrated to inhibit β-D-galactosidase.[50]

The above results may lead to the conclusion that the difference in inhibition of α-glucosidase by **6** and **7** may be due to the bigger size of **6** than **7** to interact with the active site of the enzyme. On the other hand, β-glucosidase has a different behavior where both compounds proved to be ineffective. Therefore, completely different inhibition mechanisms seem to be operative as reported earlier. Further studies with other enzymes and inhibition analyses are needed in order to clarify the structural requirements in the skeleton of the inhibitor.

ACKNOWLEDGMENTS

The authors are indebted to Prof. Dr. Richard R. Schmidt, Fakultät für Chemie, Universität Konstanz, Germany and Dr. Taha Zaghlol, Biotechnology Department, Institute of Higher Studies and Research, Alexandria University, Egypt for their valuable help. The support from AvH and DFG is highly appreciated.

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Received March 11, 2002
Accepted July 19, 2002