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INHIBITION OF β-*N*-ACETYLGLUCOSAMINIDASE BY GLYCON-RELATED ANALOGUES OF THE SUBSTRATE

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Inhibition studies on β-*N*-acetylglucosaminidase (EC 3.2.1.30) of widely differing origins (animal, plant, fungus) were carried out with *N*-acetylglucosaminono-1,5-lactone (**1**), *N*-acetylglucosaminono-1,5-lactam (**2**), 1,5-imino-*N*-acetylglucosaminitol (**3**), and *N*-acetylglucosaminono-1,5-lactone oxime (**4**). The inhibition was competitive in all cases, and K_i values were generally in the range of 0.15–2 μM, except for the fungal enzyme (5–20 μM). To assess the kinetics of enzyme-inhibitor complex formation, continuous enzyme activity monitoring was done with 3,4-dinitrophenyl-β-*N*-acetylglucosaminide as the substrate. A slow approach to the binding-equilibrium in the time scale of minutes could not be observed with any of the inhibitors tested (**1–4**). The results are evaluated as to the bearing of the enzyme source on best performance of the test compounds, the sub-type of inhibition mechanism is discussed, and suggestions are made for further analogue syntheses as well as potential applications of **1–4** (particularly the *O*-phenylcarbamoyl derivative of the latter) in biological and medical research.

KEY WORDS: β-*N*-Acetylglucosaminidase, inhibition, aldonolactone, aldonolactone oxime, aldonolactam, 1,5-dideoxy-1,5-iminoalditol.

ABBREVIATIONS: DNP-GlcNAc, 3,4-dinitrophenyl-β-*N*-acetylglucosaminide; β-GlcNAc'ase, β-*N*-acetylglucosaminidase (EC 3.2.1.30); GlcNAc-lactam, *N*-acetylglucosaminono-1,5-lactam; GlcNAc-lactone, *N*-acetylglucosaminono-1,5-lactone; GlcNAc-oxime, *N*-acetylglucosaminono-1,5-lactone oxime; GlcNAc-PC-oxime, *N*-acetylglucosaminono-1,5-lactone *O*-(phenylcarbamoyl)oxime; imino-GlcNAc, 2-acetamido-2-deoxy-nojirimycin; imino-GlcNAc'itol, 1,5-dideoxy-1,5-imino-*N*-acetylglucosaminitol; NP-GlcNAc, 4-nitrophenyl-β-*N*-acetylglucosaminide.

INTRODUCTION

β-*N*-Acetylglucosaminidases (β-GlcNAc'ases) appear to be of ubiquitous occurrence in nature and, accordingly, are assigned an array of largely different functions. In micro-organisms β-GlcNAc'ase represents part of the enzyme systems responsible for cell wall degradation during growth,[1,2] in plants it is of functional significance in the mobilisation of storage compounds during seed germination,[3] in insects it performs a developmental function,[4] and in chordates it appears indispensable for sperm-to-egg

* Correspondence.

surface binding.[5] In Man, finally, β-GlcNAc'ase plays an essential role, e.g., in renal dysfunction,[6] in the invasiveness of tumor cells,[7] and the expression of certain types of leukemia.[8] Specific inhibitors of β-GlcNAc'ase are, therefore, of interest as potential antimicrobials, insecticides, antifertility agents, and anticancer drugs.

Known β-GlcNAc'ase inhibitors on which to base further synthetic approaches are GlcNAc-based 1,5-iminoaldose,[9] the corresponding lactone (**1**)[10] (for earlier citations, see ref. 11); GlcNAc-lactam (**2**),[12] imino-GlcNAc'itol (**3**)[9,13,14] and GlcNAc-oxime (**4**).[10] With corresponding K_i values being at variance by factors of about 1000, 450, 50, 7000 and 100 for the iminoaldose and **1–4**, respectively, it is, however, not clear to what extent the disparate data indeed reflect genuine differences in inhibitory potency of the compounds tested or are due to the different organismic origins of the GlcNAc'ases or to variation in experimental conditions.

Besides yielding a guideline for the rational design of chemicals of potential use as agricultural or chemotherapeutic agents (see above), the availability of specific β-GlcNAc'ase inhibitors may be valuable also for studies into its mechanism of hydrolysis. The generally strong inhibition of the enzyme by compounds **1**, **2** and **4** and the relatively high K_m/K_i ratios observed (see e.g., ref. 10) are thought to be due to their resemblance to the pyranosyl cation-like geometry of the substrate approaching the transition-state[11,15] (see however, ref. 16). Nevertheless, there could be slight differences in their primary site of attack, since—in contrast to the "classical" situation with fast-binding competitive inhibitors—a slow establishment of the enzyme-inhibitor binding equilibrium (*cf* ref. 17) has been reported for imino-GlcNAc and the lactone **1**,[9,11,12] but not for **2** and **3**. Data are not available for **4** in this respect.

This report presents comparative data on the inhibitory potency of **1–4** with β-GlcNAc'ases of largely different origins—all tested under identical experimental conditions—and on the time-course of enzyme inhibition by these compounds.

MATERIALS AND METHODS

Enzyme Sources

β-*N*-Acetylglucosaminidase (β-GlcNAc'ase) from bovine kidney and *Canavalia ensiformis* were purchased from Sigma Chemical Co. (Lot 109F8090 and Lot 49F9641, respectively), whereas the soluble enzyme from *Mucor rouxii* ATCC 24905 was isolated as described earlier.[10] Specific activities were 150, 800, and 0.1 μkat/g, respectively.

End-point Enzyme Activity Determination and Establishment of K_i

The assay mixture consisted of 100 μl enzyme preparation (50–150 nkat/l), 25 μl substrate solution (NP-GlcNAc), and 25 μl inhibitor solution (or buffer alone), each component having been dissolved in 50 mM citrate or phosphate buffer. The assay pH was selected either according to the optimum as given elsewhere (bovine kidney, citrate pH 4.25;[18] *C. ensiformis*, citrate pH 5.0),[19] or it corresponded to the experimentally determined optimum (*M. rouxii*, phosphate pH 6.5).[20] After incubation at 37°C for 30 min the reaction was terminated by addition of 150 μl of saturated sodium tetraborate solution, and the absorbance measured at 405 nm, using a

microplate reader (Anthos Labtec Instruments, Austria). Assay mixtures treated with this borax solution at zero incubation time served as blanks. Catalytic activity concentrations (nkat/l) were calculated using a calibration curve established with 4-nitrophenolate. For each test compound, the measurements were done in four replicates at 8–10 inhibitor concentrations and three different substrate concentrations (0.5, 1 and 2 × K_m). A microcomputer modeling (χ^2-method) for competitive inhibition was carried out to establish the best-fitting K_i (for details, see ref. 10).

Continuous Enzyme Activity Monitoring

To start the measurement, 100 μl of a solution containing DNP-GlcNAc (prepared according to Yagi *et al.*)[21] and inhibitor was added to 200 μl enzyme preparation (50–300 nkat/l); 50 mM phosphate pH 6.5 served as assay buffer. Substrate concentrations were varied in the range of 0.5–2 × the corresponding K_m for NP-GlcNAc. The incubation was performed at room temperature in a microplate reader assembly (as above), which allowed measurements of A_{405} every 8 s. The amount of 3,4-dinitrophenolate released was calculated from a calibration curve.

Inhibitors

2-Acetamido-2-deoxy-D-glucono-1,5-lactam (GlcNAc-lactam) was synthesised as described by Petursson *et al.*,[22] 2-acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol (imino-GlcNAc'itol) according to Fleet *et al.*,[13] and 2-acetamido-2-deoxy-D-glucono-1,5-lactone (GlcNAc-lactone) according to Pravdic and Fletcher.[23] (*Z*)-2-Acetamido-2-deoxy-D-gluconhydroximo-1,5-lactone (GlcNAc-oxime) was purchased from CarboGen Laboratories AG, CH-8057 Zürich, Switzerland.

RESULTS

Catalytic activities were determined of β-GlcNAc'ases from bovine kidney, *C. ensiformis*, and *M. rouxii* in the presence of compounds **1–4** (for structures, see Figure 1). They all caused an inhibition which was purely of the competitive type. Best-fitting K_i constants, accounting simultaneously for the three substrate concentrations applied, were, therefore, calculated using the mathematical model for substrate competitivity. The results are presented in Table 1.

To investigate the kinetics of enzyme-inhibitor complex formation with **1–4** and the *O*-(phenylcarbamoyl)oxime of the latter (GlcNAc-PC-oxime), a substrate had to be used which allowed on-line detection of aglycon liberation. In order to combine the conveniences of visible light photometry and multiple sample measurements in a microplate reader, DNP-GlcNAc was chosen. Because of the relatively low pK_a of 3,4-dinitrophenol (pK_a 5.4),[24] its quantitative detection in the deprotonated state is only possible about as far down as pH 6.5. Experiments were, therefore, only carried out with the *C. ensiformis* and the *M. rouxii* enzyme, at inhibitor concentrations of up to 20 × the corresponding K_i. The initial non-resolved time in the method used

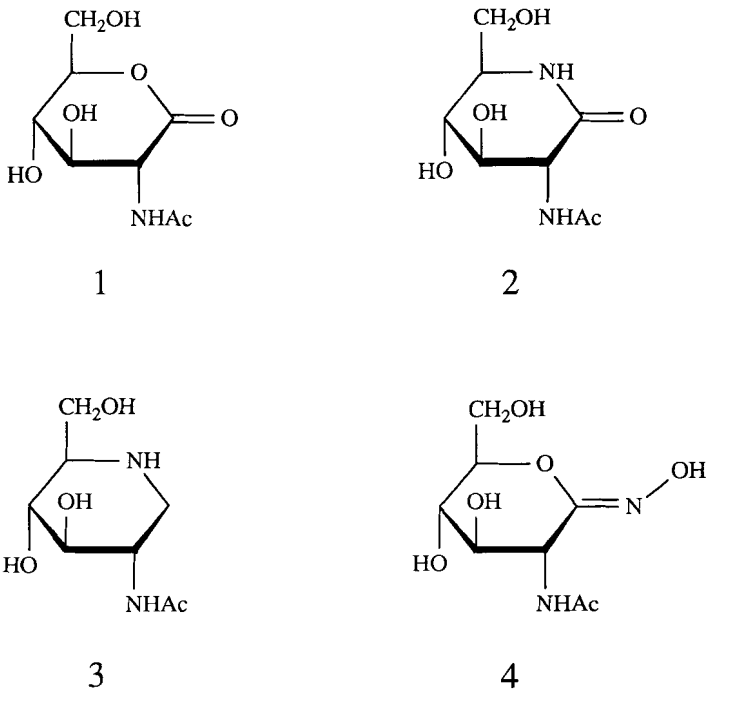


Figure 1 Structural formulae of compounds tested for inhibitory action on *β*-*N*-acetylglucosaminidase. **1**, *N*-Acetylglucosaminono-1,5-lactone; **2**, *N*-acetylglucosaminono-1,5-lactam; **3**, 1,5-dideoxy-1,5-imino-*N*-acetylglucosaminitol; **4**, *N*-acetylglucosaminono-1,5-lactone (*Z*)-oxime.

was about 15 s and included the pipetting and mixing processes. The data for the inhibition kinetics of the plant enzyme by the iminoalditol **3** and oxime **4** are presented (Figure 2), which are, however, qualitatively representative for all of the cases tested. A reproducible deviation from a time-linear type of product accumulation was not observed in any of the experiments.

Table 1 Inhibitor constants of *β*-*N*-acetylglucosaminidase from various origins for GlcNAc-based aldono-1,5-lactone (**1**), aldono-1,5-lactam (**2**), 1,5-dideoxy-1,5-iminoalditol (**3**) and aldono-1,5-lactone oxime (**4**) with 4-nitrophenyl-*β*-*N*-acetylglucosaminide as the substrate. The values are given ±SD for N = 3–6 experiments

		K_i [μM] with enzyme from		
Compound		bovine kidney	*C. ensiformis*	*M. rouxii*
GlcNAc-lactone	(**1**)	0.16±0.02	0.31±0.04	14±6
GlcNAc-lactam	(**2**)	1.80±0.30	8.97±0.60	7.22±0.9
Imino-GlcNAc'itol	(**3**)	1.70±0.10	0.36±0.06	8.25±0.9
GlcNAc-oxime	(**4**)	0.45±0.15	0.62±0.06	21.5±4

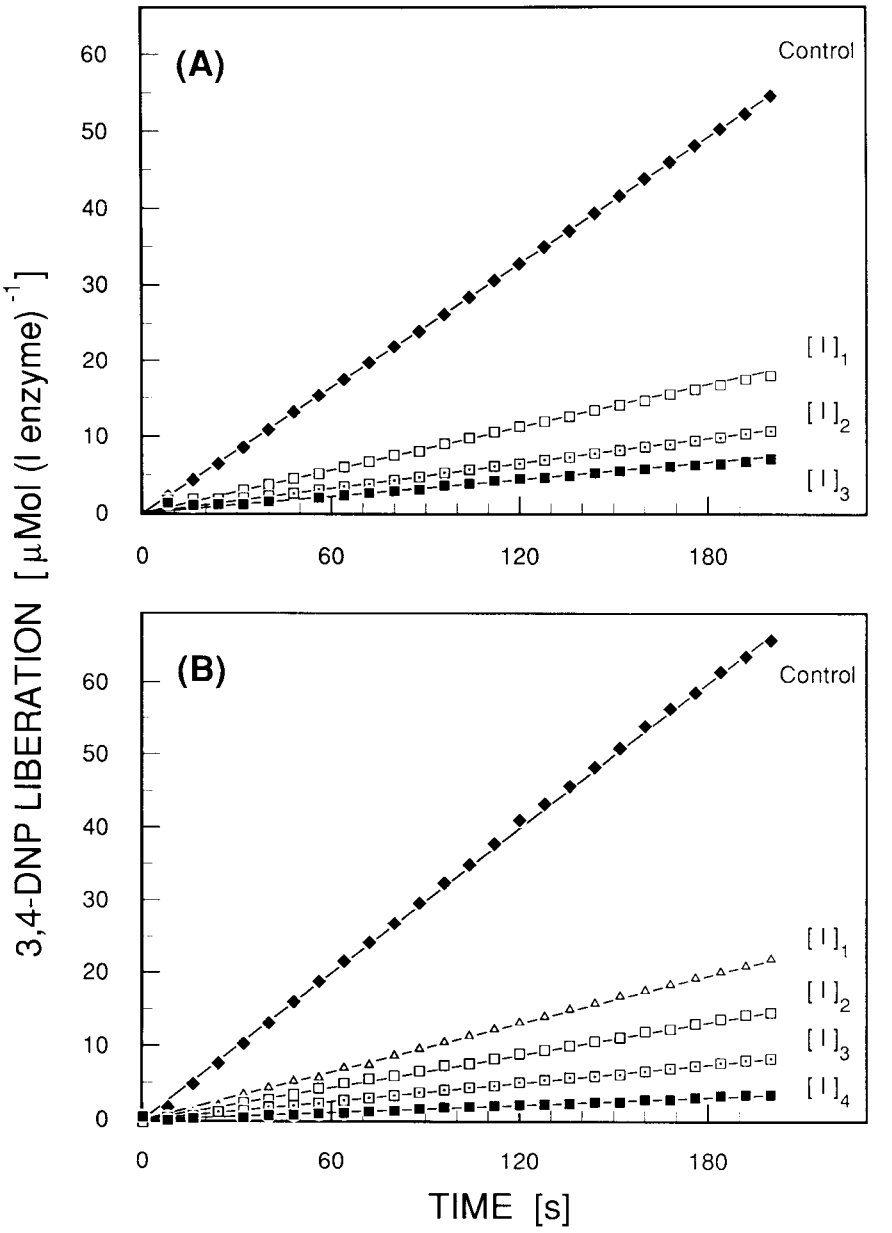


Figure 2 Time-course of 3,4-dinitrophenolate (3,4-DNP) liberation from DNP-GlcNAc by *C. ensiformis* β-*N*-acetylglucosaminidase in presence or absence of the inhibitors *N*-acetylglucosaminono-1,5-lactone oxime (A) and 1,5-dideoxy-1,5-imino-*N*-acetylglucosaminitol (B). Inhibitor concentrations were in (A), $[I]_{1-3} = 50, 100, 200\ \mu M$, respectively; and in (B), $[I]_{1-4} = 0.5, 1, 2, 7\ \mu M$, respectively.

DISCUSSION

K_i Values and Potential Applications

As compared to the bovine and plant β-GlcNAc'ases, the fungal enzyme displays relatively high K_i values for any of the test compounds; they are all in the 7–22 μM range (Table 1). This observation might be construed as evidence for a discrepancy in the transition-state of the rate-limiting step of the β-GlcNAc'ases concerned. The difference noted is, however, rather a consequence of the unusually high affinity of the fungal enzyme for the phenylglucosaminide substrate ($K_m = 130$ μM, as compared to 620 and 1500 μM for the plant and animal enzymes, respectively),[10] the aglycon of which, thus, affording additional binding energy (the K_m for the natural substrate, chitobiose, is 1.2 mM).[25] Indeed, when the oxime inhibitor **4** is derivatised to encompass an analogous aryl partial structure,[10] the difference in susceptibility between the fungal enzyme and the other β-GlcNAc'ases studied is abolished. The fact that the K_i's (GlcNAc) are similarly high in all cases, namely 4–6 mM (ref. 9, and M. Horsch & D.M. Rast, unpublished), is in agreement with this interpretation.

Whereas the K_i observed for the lactone **1** with the *M. rouxii* enzyme is similar to that of *Aspergillus niger* β-GlcNAc'ase,[25] values for the lactam **2** and oxime **4** are somewhat lower than found earlier with β-GlcNAc'ase of the latter fungus (50 μM).[26] With about 1 mM as observed also before,[9] the K_i of the *A. niger* β-GlcNAc'ase for imino-GlcNAc'itol (**3**) stands, however, largely apart. The *A. niger* enzyme appears, indeed, to be quite generally of a very low sensitivity to β-GlcNAc'ase inhibitors (no inhibition at all was noticed by Fleet *et al.*)[27]—the same commercial preparation was used by all authors cited—since also inhibition with GlcNAc-PC-oxime was moderate ($K_i = 800$ nM as compared to 40 nM for the *M. rouxii* enzyme)[25] and even poor with the corresponding phenylsemicarbazone (6000 *vs* 130 nM for the bovine kidney enzyme).[28] The further use of this apparently fairly crude *A. niger* product (from the manufacturer's information isolated according to Bahl and Agrawal)[29] for the search of potential β-GlcNAc'ase inhibitors is, therefore, not advisable.

With the exception of **2**, inhibition by **1–4** of the *C. ensiformis* β-GlcNAc'ase was better than with the *M. rouxii* enzyme. The K_i found here for the plant enzyme for imino-GlcNAc'itol (**3**) is intermediate between those reported earlier from the same source[27] and the enzyme from *Zea mays*.[14] The inhibition constant of the bovine kidney enzyme for **3** was very similar to values found before[9,27] and somewhat lower than for β-hexosaminidase from human liver, which displayed a K_i value of *ca* 5 μM.[22] With a corresponding value of 1.8 μM, the inhibitory potency of the lactam **2** with the bovine kidney enzyme is almost the same as with the human liver enzyme.[22]

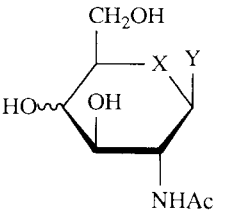
Taking the inhibitory efficacy of the GlcNAc-lactone as the reference, the GlcNAc-oxime and the imino-GlcNAc'itol perform about equally well in all tests, except in the case of the latter with the animal enzyme. The GlcNAc-lactam, however, is about 10–30 times less potent—except in the case of the fungal enzyme. Nevertheless, with $K_i = 160$ nM the lactone itself does not represent the best β-GlcNAc'ase inhibitor described previously, as the lowest values reported are 40 and 1 nM, for GlcNAc-PC-oxime (*cf* **4**) and 2-acetamido-2-deoxynojirimycin (imino-GlcNAc), respectively.[9,10] Imino-GlcNAc'itol (**3**) has recently been shown to inhibit β-hexosaminidase-mediated degradation of the extracellular matrix in human ovarian carcinoma,[30] and to, thus, represent a potential antimetastatic drug.[7] In view of the higher β-GlcNAc'ase inhibitory potency of **1**, **4** and, particularly, the phenylcarbamoyl derivative of the latter, it is proposed to test the oximes for their efficacy also *in vivo*,

using the same cell-line. Because of its low stability in aqueous solution,[11] the use of **1** for the same purpose does not, however, appear to be promising. Considering the multiple functions assigned to β-GlcNAc'ase in micro-organisms, animals and Man (see Introduction), these inhibitors suggest themselves as tools also in various other areas of biological and medical research.

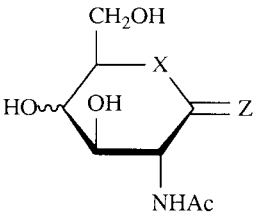
Mechanistic Interpretation of Binding Kinetics and Suggestions for Future Analogue Syntheses

The inhibitors in question are to be classified substrate-competitive and, thus, the formation of the enzyme-inhibitor complex must necessarily be a reversible process. A slow approach to the inhibitor-enzyme binding-equilibrium in the time scale of minutes may sometimes occur as a result of very tight binding, to the extent that steady-state kinetic conditions are no longer met, and/or in combination with slow turnover rates in complex formation (*cf* ref. 17). In contrast to the reported case of a slow inhibition with bovine β-GlcNAc'ase (for citations, see Introduction), the same could not be observed with any of the inhibitors tested, using the *C. ensiformis* as well as the *M. rouxii* enzymes. Furthermore, this was not apparent even in the case of the very strong inhibition of the fungal enzyme with GlcNAc-PC-oxime ($K_i = 40$ nM), where a deviation from steady-state conditions might have been most likely to occur—if at all. These findings, therefore, suggest, that slow-binding of potent inhibitors is not a conspicuous and conserved feature of β-GlcNAc'ases and may be due solely to minor alterations near the active site.

Considering the substrate specificity of β-GlcNAc'ase (EC 3.2.1.30) and β-*N*-acetylhexosaminidase (EC 3.2.1.52), both acting on *N*-acetyl-β-D-glucosaminides as well as *N*-acetyl-β-D-galactosaminides, and accounting for the structures of all potent inhibitors of them (K_i's in the μM range or below), the following approach to the design of potential further inhibitors appears to be most promising. It is based on the Markush formulae **I** and **II**, as outlined.



I



II

Formula **I** represents potential substrates, with X = O and Y = OR, as well as the inhibitors imino-GlcNAc'itol (**3**: X = NH, Y = H), imino-GlcNAc (X = NH, Y = OH),[9,12] *N*-alkylimino-GlcNAc'itol† (X = $N(CH_2)_5COOH$, Y = H),[12] dimethyliminio-GlcNAc'itol

† *Systematic names*: *N*-alkylimino-GlcNAc'itol, *N*-(5-carboxypentyl)-1,5-dideoxy-1,5-imino-*N*-acetylglucosaminitol; 1-amino-GlcNAc, 1-amino-β-*N*-acetylglucosaminide; dimethyliminio-GlcNAc'itol, *N,N*-dimethyl-1,5-dideoxy-1,5-iminio-*N*-acetylglucosaminitol; GlcNAc-phenylsemicarbazone, *N*-acetylglucosaminono-1,5-lactone 4-phenylsemicarbazone; NAc-castanospermine, 6-acetamido-6-deoxy-castanospermine.

($X=N^+(CH_3)_2$, $Y=H$),[12] NAc-castanospermine ($X=N(CH_2)_2$ linked to C(6), $Y=H$),[31] the much less efficient 1-amino-GlcNAc ($X=O$, $Y=NH_2$),[9,32] and arylthio-β-*N*-acetylglucosaminides ($X=O$, $Y=SC_6H_4NO_2$ or $Y=SC_6H_5$).[33] Formula **II** represents the transition-state approximates (*cf* Introduction) GlcNAc-lactone (**1**: $X=Z=O$), GlcNAc-lactam (**2**: $X=NH$, $Z=O$), GlcNAc-oxime (**4**: $X=O$, $Z=NOH$), GlcNAc-PC-oxime ($X=O$, $Z=NOCONHC_6H_5$)[10] and GlcNAc-phenylsemicarbazone ($X=O$, $Z=NNHCONHC_6H_5$).[28] Not included in the list of known inhibitors, since not covered by **I** or **II**, is the GlcNAc-1,4-lactone[34] with a *ca* 10-fold lower efficacy than the corresponding 1,5-lactone. Common to all type **I** and type **II** inhibitors is the D-gluco configuration, but the corresponding compounds with D-galacto configuration might be inhibitors as well.

Further desirable structural analogues to be tested as modulators of the two enzyme activities concerned could include variations of X in **I** and **II** that encompass additional N-alkyl, N-aryl and N-acyl groups as well as sulfur—also as a means to study the steric restrictions and electronic contribution of the ring heteroatom to binding to the active site (*cf* ref. 11). Furthermore, regarding **I**, probing variations in Y, particularly non-hydrolysable C-glycosides, might provide additional insight into the substrate binding mode. With respect to **II**, further variation of Z by even more lipophilic extensions of oximes or hydrazones than just the phenylcarbamoyl moiety might be of heuristic value towards understanding the interaction of *N*-acetylhexosaminidases with aglyca (*cf* ref. 10, 28).

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