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N^1-Alkyl-D-gluconamidines: Are they 'perfect' mimics of the first transition state of glucosidase action? [1]

Günter Legler *, Marie-Therese Finken

Institut für Biochemie der Universität Köln, Otto-Fischer-Str. 12-14, D-50674 Cologne, Germany

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Abstract

The inhibition of four β-glucosidases of plant, fungal, and mammalian origin by N^1-butyl- and N^1-dodecyl-D-gluconamidine was determined. Comparison with the inhibition by the corresponding *N*-alkyl-D-glucosylamines revealed that the strongly basic amidines (pK_a 10.8) were at the most 10-times more inhibitory than the weakly basic glucosylamines (pK_a 6.5). The small enhancement of inhibitory potency, resulting from transforming the tetrahedral C-1 geometry of the glucosylamines to the planar sp^2-geometry of the amidines, was ascribed to the inability of the fully protonated amidines to function as hydrogen bond acceptors with the catalytic acid of the enzyme. Additional evidence for the importance of a hydrogen bond for strong inhibition came from the comparison of K_I-values of the weakly basic 5-amino-5-deoxyhexopyranoses and 1,5-iminohexitols with those of the corresponding glyconamidrazones (pK_a 8.4), which also have a planar C-1 geometry but are largely protonated under the assay conditions and which had similar or up to 10^4-times larger K_I-values than the former. Transition state resemblance was judged from the ratio K_S(alkyl β-glucoside)/K_I(alkyl gluconamidine) relative to the rate acceleration factor k_{cat}/k_{uncat} (Wolfenden, *Acc. Chem. Res.*, 5 (1972) 10–16). Compared to ratios of k_{cat}/k_{uncat} from $\geq 10^{11}$ to $\geq 10^{13}$, the ratios for K_S/K_I were only from 10^3 to 2×10^4 except for β-glucosidase A_3 from *Asp. wentii* which had K_S/K_I 2.8×10^6. This enzyme differs from the others by being strongly inhibited by cationic glycon and substrate analogues rather than by basic ones. The pH-dependence of $1/K_I$ and the 'slow' approach to the inhibition is discussed with respect to transition state resemblance. © 1996 Elsevier Science Ltd.

* Corresponding author.

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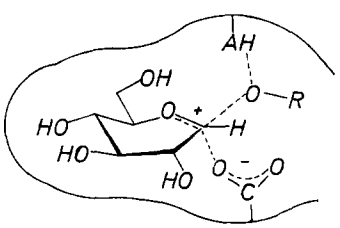
1. Introduction

Most glycosidases catalyze the hydrolysis of their substrates with retention of configuration, i.e. the product sugar is released with the same anomeric configuration as that of the substrate glycoside. This is most likely to be achieved by a two-step double displacement mechanism where, in the first step, the aglycon is released with the simultaneous formation of a glycosyl enzyme intermediate with inverted anomeric configuration. In the second step, the intermediate is cleaved by a water molecule with release of the sugar in its original configuration. Aside from possibly rate-limiting conformational alterations of the enzyme, there are thus two transition states to be passed on the pathway from substrate to products. Generally, the observed rate depends mainly on that of the first step but there are many examples, especially with glycosides having an aglycon of high leaving-group propensity, where deglycosylation of the intermediate is rate-limiting.

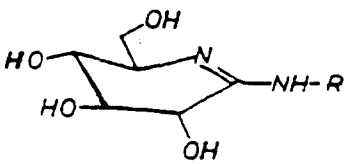
The transition state for the first step is currently described by a model (see refs [1,2] for reviews) where the hexose is deformed from its ground state chair conformation toward a structure flattened at C-1. Concomitant breaking of the glycosidic bond is aided by a strong hydrogen bond to the glycosidic oxygen leading to proton transfer in the transition state and by the stabilization of a (partial) positive charge on C-1 by a closely positioned carboxylate group (Scheme 1). Departure of the aglycon, and formation of a glycosyl ester with the carboxylate, would complete the first step. The catalytic cycle is completed by hydrolysis of the glycosyl enzyme where the orientation and deprotonation of the water molecule in the second transition state also proceeds with inversion, causing the sugar to be released in the original anomeric configuration. Note that the hydrolysis of the glycosyl enzyme differs from ester hydrolysis in solution in that the water molecule reacts with the alkyl carbon rather than with the carboxyl C=O group.

As the aglycon is part of the first transition state, a good imitation should have, in addition to features of the putative glycosyl oxocarbonium ion, a substituent at C-1 for interactions with the aglycon binding site. With most glycosidases, these interactions appear to be not very specific so that simple alkyl residues might be a convenient choice for exploratory studies. In order to obtain information on the combined effects of a planar geometry and a strongly basic centre at C-1 and a substituent to interact with the



Scheme 1. Paradigmatic model for the first transition state of enzymic β-glucoside hydrolysis.

aglycon site, we have synthesized N^1-butyl- and N^1-dodecyl-D-gluconamidine (**1** and **2**, see preceding communication [3]) and studied their inhibitory activity with one α- and four β-glucosidases. Our aim was to evaluate the resemblance of these inhibitors with the first transition state by comparing their inhibition constants K_I with the dissociation constants of the enzyme substrate complex and the rate enhancement factors over the uncatalyzed reaction according to Wolfenden [4]. Inhibition by the corresponding *N*-alkyl-D-glucosylamines was included to judge the effects of geometry of C-1 (trigonal versus tetrahedral) and basicity (pK_a 10.5 versus pK_a 6.0 [5]).



1 R = butyl
2 R = dodecyl

The enzymes listed in Table 1 were selected for testing the inhibitory potential of **1** and **2** because a large body of published kinetic data and studies with other glycon and substrate related inhibitors would facilitate the interpretation of our results. α-Glucosidase from yeast was included to evaluate the effects of the anomeric specificity on the inhibition. The four β-glucosidases can be characterized as follows:

β-Glucosidase from almonds has been widely studied [7]. It is nonspecific with respect to aglycon structure, presumably because its aglycon site has to interact in succession with both the D-glucose and the mandelonitrile moiety of its natural substrate amygdalin (mandelonitrile-β-gentiobioside). Evidence for the presence of a carboxylate in close proximity to the bond cleavage site comes from studies with the active site directed inhibitor conduritol B epoxide (CBE) [8] and with basic glycon and substrate analogues [5,9]. Nevertheless, Scheme 1 appears to be inadequate to represent the transition state of the rate limiting step. Formation of a glucosyl cation-like species is ruled out by the secondary α-deuterium kinetic isotope effect $k_H/k_D = 1.01$ with phenyl β-D-glucoside [10] and Dale et al. [11] concluded from the absence of a solvent kinetic isotope effect that there is no proton transfer to the glycosidic oxygen in the first irreversible step. From the effects of leaving-group acidity on k_{cat}/K_M, the latter authors postulated the nucleophilic attack of the active site carboxylate at C-1 with formation of an α-glycosyl enzyme. This step would be rate limiting with leaving groups of $pK_a > 7$, whereas with the more acidic ones the rate would be limited by deglucosylation. A partially rate limiting conformational change cannot be ruled out, however, because *o*- and *p*-substituted phenyl glucosides with very similar pK_as differ up to tenfold in k_{cat} [12].

β-Glucosidase A_3 from *Asp. wentii* shows a strong preference for cellobiose; aliphatic and aromatic β-glucosides are cleaved up to 20-times less efficiently [13,14]. It differs from most other glycosidases in its sigmoidal pH-dependence of k_{cat} for substrate hydrolysis and inactivation by CBE, indicative of an acidic group with pK_a

5.4. Ionization of the aspartate labelled by 2-deoxyglucosides [15] and CBE [16] does not show up in the pH-rate curves. A glycosyl cation-like transition state is documented by the α-deuterium isotope effect $k_H/k_D = 1.14$ [14]. The pH-dependence of $1/K_I$ of cationic glycon and substrate analogues is sigmoidal, indicative of an anionic group with pK_a 5.6. This enzyme belongs to the small group of glycosidases which are inhibited much better by the cationic (protonated) form of nitrogen containing sugar analogues than by the basic form [14].

Mammalian *lysosomal* β-glucosidases have an aglycon site adapted to their natural substrate, β-glucosylceramide, i.e. an extended cleft to accommodate, in addition to the three hydrophilic groups of acyl sphingosine, at least eleven CH_2-groups (see ref. [17] for a review). Hydrophobic interactions make a substantial contribution to the inhibition by long chain *N*-alkyl-1-deoxynojirimycin derivatives and glucosylamines [18]. β-Glucosylceramide and 4-alkylumbelliferone β-glucosides, which differ widely in leaving-group propensity of the aglycon, are hydrolysed with very similar values of k_{cat} [19]. This, and an activation by higher alcohols correlating with efficient transglucosylation [20], point to deglucosylation of a glucosyl enzyme intermediate as rate limiting step. This means that the Michaelis constant K_M is smaller than the dissociation of the enzyme substrate complex K_S. Long chain alkyl β-glucosides, on the other hand, are cleaved at least ten-times more slowly, i.e. the first bond breaking step is probably rate limiting and K_M can be taken as a good approximation to K_S.

The mammalian *cytosolic* β-glucosidases, also called non-specific β-glucosidases/-galactosidases [21], differ from the lysosomal ones by their lack of specificity with respect to the C-4 hydroxyl group and by being inhibited rather than activated by anionic lipids like taurodeoxycholate and acidic phospholipids. Their aglycon site is also hydrophobic but without a hydrophilic region adjacent to the glycosidic bond [22]; their natural substrates are unknown. Aromatic β-gluco- and -galactosides are 'good' substrates, long aliphatic ones being hydrolyzed $\sim$ 50-times more slowly [22]. A major mechanistic difference from the lysosomal enzyme, and from almost all other β-glucosidases, is their resistance to inactivation by CBE [23]. Studies with the guinea pig enzyme have shown that deglucosylation of an intermediate is rate limiting in the hydrolysis of 'good' substrates [24].

2. Results and discussion

The inhibition constants K_I for the two N^1-alkyl-D-gluconamidines **1**, **2** and for the two types of reference compounds are listed in Table 1. The contribution of the alkyl substituents to the inhibition could be estimated from a comparison with the K_I-values of D-gluconamidrazone (Table 2, last column) which has small, polar NH_2-substituents on the exocyclic nitrogen. The comparison shows that the butyl group made an only marginal contribution to the inhibition of the enzymes from almonds and *Asp. wentii*. The cytosolic β-glucosidase, on the other hand, was inhibited about 150-times better by **1**. This, and the large contribution by the dodecyl group, confirmed earlier conclusions about an adaptation of the aglycon site of the two mammalian enzymes to extended hydrophobic substrates.

Table 1
Inhibition of β- and α-glucosidases by the N^1-alkyl-D-gluconamidines **1** and **2** and by the corresponding *N*-alkyl-D-glucosylamines and β-D-glucopyranosides. (K_I-values in μM at pH 6.0 except where indicated otherwise; K_S / K_I refers to β-glucosides and amidine type inhibitors)

Enzyme	**1**	C_4-Glucosylamine	C_4-β-Glucoside	K_S / K_I
β-Glucosidases				
Sweet almonds	4.2	12	20000	4700
Asp. wentii				
pH 5	0.013			
pH 6	0.0043	0.066	12000	2.8×10^6
pH 7	0.0030			
Bovine, cytosolic, pH 7	0.13	1.3	210	1600
α-Glucosidase				
Yeast	9.0	–	8000 [a] 80000 [b]	~1000
β-Glucosidases	**2**	C_{12}-glucosylamine	C_{12}-glucoside	
Bovine, cytosolic, pH 7	0.00020 [c]	0.0027	0.17	850
Bovine, Lysosomal, pH 5	0.0007	0.0005	15	22000

[a] K_I for ethyl 1-thio-α-D-glucopyranoside [6].
[b] K_M for maltose [6].
[c] Competitive component of mixed competitive/non-competitive inhibition.

In spite of their greater similarity with the putative glucosyl cation-like transition state, the gluconamidines turned out to be only slightly better inhibitors than the corresponding *N*-alkyl-D-glucosylamines. This was seen with all β-glucosidases studied here, even though they differ considerably with respect to details of mechanism. Detrimental steric effects caused by an unfavourable orientation of the alkyl substituent are probably not responsible for the small contribution of sp^2-geometry to inhibitory potency. The predominant tautomeric form of type **1**, **2** amidines has an endocyclic double bond as shown [25]; free rotation of the –NH–R group will thus not be impaired.

A possible explanation for the small enhancement of the inhibitory potency of the type **1**, **2** compounds by sp^2-hybridization at C-1 and the positive charge could be their fully protonated state under the assay conditions caused by their high basicity (pK_a 10.8 [3]). Studies with various types of basic glycon derivatives [1,2] have shown that most glycosidases are inhibited much better by the unprotonated form of the inhibitor, provided the protonated form has $pK_a > 5$. Only a few enzymes like β-glucosidase A_3 from *Asp. wentii* are strongly inhibited by protonated or permanently cationic glycon derivatives, e.g. glycosylpyridinium salts or *N*,*N*-dimethyliminoalditols. For the majority of glycosidases, this could be taken as evidence for a substantial contribution to the binding energy by a hydrogen bond with the catalytic acid –AH (Scheme 1) as donor and the basic nitrogen of the inhibitor as acceptor. If proton donation is considered to be complete, ion-pair formation would result which could also contribute to the binding energy. The latter model was favoured by BeMiller et al. [31] to explain the inhibition

Table 2
Glycosidase inhibition by sugar analogues of different C-1 geometry and basicity. (K_I-values in μM; pK_a-values are for the *gluco*-configurated compounds; data without reference are from this study)

Enzymes	Hexono-1,5-lactone $pK_a < 0$	5-Amino-5-deoxy-hexopyranose pK_a 5.3	1,5-Dideoxy-1,5-iminohexitol pK_a 6.3	Hexono-amidrazone pK_a 8.4
β-Glucosidases				
Sweet almonds [a], pH 5.0	5.0	1.3 [b]	48	4.7 [b] 8.4 [25]
Asp. wentii, pH 5.0	6.5	0.07 [b] [14] 0.047 [c]	0.30 [b]	0.031 [b]
Bovine, lysosom., pH 5.0	1.8	0.8 [b]	42	3.3 [b]
Bovine, cytosol., pH 7.0	15 [23] [a]	42 [b]	210 [18]	19 [b]
α-Mannosidase				
Jack beans, pH 5.0	120 [26]	1.2 [b] [27]	68 [27]	0.17 [25]
α-Galactosidase				
Coffee beans, pH 6.0	–	0.0007 [b] [28]	0.0016 [b] [28]	8.3 [25]
α-L-Fucosidase				
Bovine, pH 6.0.			0.0027 [b] [29]	0.82 [30]

[a] Dale et al. [9] have probably studied a different isoenzyme (Sigma, Type I) because they report K_I-values for α / β-D-glucose, 1-deoxynojirmycin and D-glucono-1,5-lactone which are from 4- to 40-times larger than found with the Sigma preparation used in this study.
[b] Slow approach to the inhibition equilibrium.
[c] Calculated with the concentration of protonated inhibitor.

characteristics of β-galactosidase from *E. coli* by *N*-substituted β-D-galactosylmethylamines of pK_a 8.0 to 9.7. It fails, however, to account for the strong inhibition of this enzyme by *N*-alkyl-D-galactosylamines [32] because ion-pair formation between an acid with pK_a 8.3 and a base with pK_a 6.2 appears unlikely. Also, there is a conceptual problem with the ion-pair model when applied to glycosidases with catalytic acids –AH having pK_a-values near the pH of the assay buffer. A sufficiently large proportion of $-A^-$ would be available to bind cationic inhibitors. Only with enzymes where –AH is a cationic acid like protonated histidine, would the ion-pair have to be formed by proton transfer within the active site [5].

Additional support for the importance of hydrogen bonds with a substituent on C-1 or ion-pair formation within the active site comes from a comparison of sugar derivatives having different basicity and C-1 geometry (Table 2). Published data and the results of this study demonstrate that nojirimycin (1,5-deoxy-1,5-imino-D-glucopyranose, pK_a 5.6) and its *galacto*-analogue are similar or even better inhibitors than the corresponding amidines and amidrazones, even though the nojirimycins have the tetrahedral sp^3- rather than the planar sp^2-geometry at C-1. β-Glucosidase A_3 from *Asp. wentii* is inhibited by protonated nojirimycin and D-gluconamidrazonium ion to the same extent because this enzyme belongs to the small group of glycosidases which have a preferential affinity for cationic glycon derivatives [14]. The only exception is seen to be jack bean α-mannosidase for which the *manno*-configurated amidrazone is a 7-times better inhibitor than *manno*-nojirimycin. We conclude that *the advantage of the glycosyl cation-like structure of the amidine and amidrazone type inhibitors is offset by their strong basicity which causes them to be fully protonated and by the inability of the inhibitor cations to act as hydrogen bond acceptors*. The up to 56-times larger inhibitory potency of the nojirimycins relative to their 1-deoxy derivatives, factors too large to be accounted for by the difference in pK_a-values, might likewise be due to the inability of the latter to form a hydrogen bond as in Scheme 1. Kajimoto et al. [33], on the other hand, conclude from free energy calculations based on contributions from ring conformations, charge–charge interactions, and hydrogen bonds with the C-2 and C-6 hydroxyl groups that electrostatic interactions with the positive charge of inhibitor and the enzyme are the most important factors for binding and recognition of half-chair inhibitors. It should be noted that hydrogen bonds, as in Scheme 1, were not included in the calculations.

Papandreou et al. [25] state that both conformational and electrostatic factors are important for active site binding but, in contrast with Kajimoto et al. [33], they argue that the sp^2-hybridization of C-1 is more important than the positive charge at this position because D-gluconamidine (pK_a 10.6) and D-gluconolactam oxime (pK_a 5.6) were of similar inhibitory potency. This finding might equally well be explained by the fact that the oxime can function as hydrogen bond acceptor whereas the amidinium ion cannot. In addition, both hypotheses advanced in refs. [25,33] fail to explain why most glycosidases [2], including the enzyme from almonds [5,9], preferentially bind the basic (unprotonated) form of the inhibitor rather than the cationic one. These hypotheses also do not account for the strong binding of the moderately basic nojirimycin and its analogues (Table 2 and ref. [2]).

The inhibition of yeast α-glucosidase by **1** (K_I 9 μM, Table 1) was very similar to the inhibition by nojirimycin (K_I 6.3 μM [1,2]) and 1-deoxynojirimycin (K_I 13 μM

[I_2] and 25 μM [34]). The data show that for this enzyme, too, the glucosyl cation-like character does not give any particular enhancement of the inhibitory potency. Comparison with *N*-butyl-D-glucosylamine was not considered meaningful because the β-configurated anomer is present in large excess [35]. The value for K_I-**1**/K_I (ethyl 1-thio-α-D-glucoside) is seen to be similar to K_S/K_M for the majority of the β-glucosidases tested here (see next section).

Transition state resemblance judged by K_S/K_I.—Based on the idea that enzymes have evolved towards an optimal complementarity to the transition state rather than to the substrate in the ground state Wolfenden [4] has derived an equation which relates rate acceleration factors with the dissociation constants of the enzyme transition state K_{TS} and that of the enzyme substrate complex K_S: $K_S/K_{TS} = k_{cat}/k_{uncat}$ where k_{cat} and k_{uncat} are the rate constants for the catalyzed and uncatalyzed reaction, respectively. K_{TS}, being a virtual dissociation constant, is not amenable to experimental determination, but one can judge the resemblance of putative transition state analogues with the transition state by comparing the ration K_S/K_I with the acceleration factor k_{cat}/k_{uncat}, i.e. for a 'perfect' transition state mimic K_S/K_I should come close to k_{cat}/k_{uncat}.

Application of this principle to glycosidases poses the following problems: the uncatalyzed rates of hydrolysis could only be measured with glucosidases having an aglycon (4-nitrophenol, 4-methylumbelliferone) with good leaving-group propensity [14]. Phenyl and methyl β-glucoside were not measurably hydrolyzed after 48 h at 110 °C. From these findings, we have to conclude that $k_{uncat} \leq 10^{-12}$ s^{-1} for alkyl β-glucosides. Values for k_{cat} for the β-glucosides listed in Table 1 can be estimated from published data as 50 ± 20 s^{-1} for the β-glucosidases from almonds [36] and *Asp. wentii* [13]; for the cytosolic β-glucosidase, we get k_{cat} 0.5 s^{-1} (C_4-) and 0.1 s^{-1} (C_{12}-β-glucoside) [22] and for the lysosomal enzyme with C_{12}-β-glucoside k_{cat} 10 ± 3 s^{-1} [17]. The acceleration factors k_{cat}/k_{uncat} thus range from $\geq 10^{11}$ with the cytosolic β-glucosidases to $\geq 10^{13}$ with the enzyme from *Asp. wentii*. Comparison of these figures with K_S/K_I (Table 1, last column) shows that this ratio is still 7 orders of magnitude lower than the acceleration factor. For enzymes like β-glucosidase from almonds, or the mammalian ones acting 'good' substrates, such estimates get even more unfavourable because the first bond breaking reaction (for which the inhibitor was modelled) may be faster than the rate limiting step (conformational change, deglycosylation) which determines the experimental value for k_{cat}.

pH-Dependence and 'slow' approach to the inhibition.—The pH-dependence of k_{cat} or k_{cat}/K_M is governed by the effect of the enzyme's ionization state on its affinity for the transition state, i.e. by the pH-dependence of $1/K_{TS}$. A good transition state mimic should thus show a pH-dependence of $1/K_I$ similar to that of k_{cat}. Inhibition measurements with **1** and **2** and three β-glucosidases gave the following results:

The inhibition of the *Asp. wentii* enzyme by **1** increased with pH (Table 1), but less than that of other cationic glycon or substrate related inhibitors [14]. Nevertheless, the pH-dependence of $1/K_I$ **1** did not resemble that of k_{cat} [13] which showed a sigmoidal increase with decreasing pH. We thus have to conclude that, in spite of the large K_S/K_I, **1** is not a transition state mimic but a cationic substrate analogue which derives its large inhibitory potency from ion-pair formation with carboxylate groups of the active site.

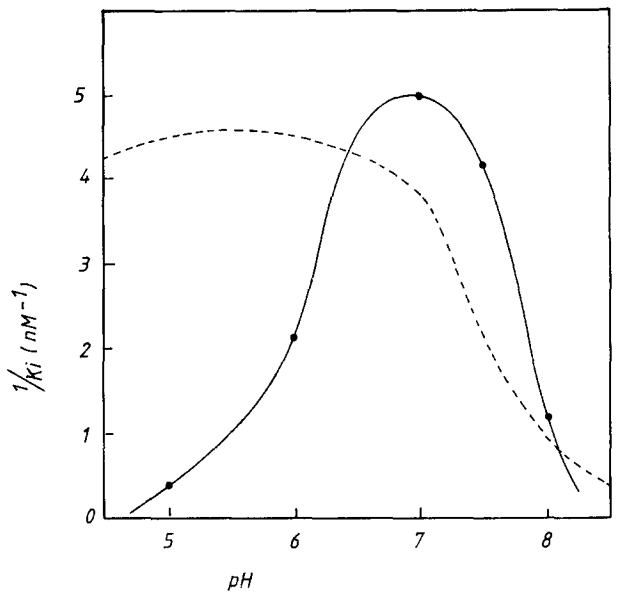


Fig. 1. pH-Dependence of $1/K_I$ for the inhibition of bovine cytosolic β-glucosidase (————) and k_{cat} for hydrolysis of 4-methylumbelliferyl β-D-glucopyranoside adapted from [22] (---).

Our results with cytosolic β-glucosidase and **2** are shown in Fig. 1. The pH-dependence of $1/K_I$ bore a faint resemblance to that of k_{cat}/K_M [22]; the main difference was a much broader profile of the latter and a shift of the maximum of $1/K_I$ to higher values of pH. The difference was even more pronounced when $1/K_I$ was compared with k_{cat} (not shown). This lack of resemblance is probably due to the fact that **2** was modelled according to Scheme 1 with dodecyl alcohol as aglycon, whereas k_{cat} refers to the hydrolysis of 4-methylumbelliferyl β-glucoside, a 'good' substrate where deglucosylation of a glucosyl enzyme intermediate is largely rate limiting [24]. Similar differences between the pH-profiles of $1/K_I$ and k_{cat} (not shown) were seen with the lysosomal β-glucosidase and **2** where the same reservations with respect to the rate limiting step responsible for k_{cat} (with 4-nonylumbelliferyl β-glucoside) have to be made.

A noteworthy point with regard to transition state resemblance is a slow approach to the inhibition equilibria, even though the binding process does not involve any covalent interactions. Except with yeast α-glucosidase, **1** and **2** showed this feature with all β-glucosidases studied here. Identical rates of substrate hydrolysis were reached, whether the inhibitor was added to a mixture of enzyme and substrate or the reaction was started by the addition of substrate to the enzyme preincubated with inhibitor. A slow onset of the steady state inhibition was also seen with C_{12}-D-glucosylamine and the mammalian lysosomal [18,37] and cytosolic β-glucosidases [22] and, where indicated, with the enzyme inhibitor combinations listed in Table 2. The rates of approach to the

final inhibition showed a roughly linear dependence on the inhibitor-concentration. This points to the rapid formation of a loose enzyme inhibitor complex with a dissociation constant $K_I' > [I]$ which then undergoes a slow conformational isomerization to a tight complex with the overall dissociation constant $K_I = K_I' k_6/(k_5 + k_6)$ where k_5 and k_6 are the rate constants for the forward and backward isomerization steps and $k_5 \gg k_6$ [38]. The phenomenon of slow inhibition has been discussed with respect to transition state resemblance of the inhibitors by Morrison and Walsh [38] and by Schloss [39]. It has been argued that such inhibitors can shed light on the structure of reaction intermediates and the transition state [38] and that slow binding and dissociation of structural analogues of highly reactive intermediates (e.g. glycosyl oxocarbonium ions in the case of glycosidases) is a consequence of keeping such intermediates bound at the active site until they have completed their normal reaction pathway [39].

Application of these arguments to glycosidase inhibitors for a detailed interpretation of the experimental data, with respect to transition state resemblance and similarity with reactive intermediates, poses a number of problems because quite different sugar and substrate analogues may exhibit slow inhibition [2]. Such analogues include weakly basic and somewhat flexible 5-amino-5-deoxyhexopyranoses and 1,5-deoxy-1,5-iminohexitols, rigid indolizine alkaloids like castanospermine and swainsonine, *N*-alkyl-glycosylamines and, as shown in this study, strongly basic glucon-amidines and -amidrazones. The latter two contrast with the others by having a C-1 with planar sp^2- rather than the tetrahedral sp^3-geometry. A few cases are known where even nonbasic hexono-lactones and -lactams exhibit slow inhibition [3,40].

The only common features for these compounds are K_I-values of 10^{-6} M and below and, except for the lactones and lactams, a basic or cationic centre at C-1. The more widespread occurrence of inhibitors that have small K_I-values and slow inhibition having an endocyclic nitrogen may reflect a greater similarity with the endocyclic delocalized positive charge of the glycosyl oxocarbonium ion.

The rates of approach to the steady state expressed as second order rate constants $k_{appr} = k_{obs}/[I]$ and corrected for the protection of the enzyme by substrate [38] ranged from 3×10^3 M^{-1} s^{-1} for **1** to 3.5×10^6 M^{-1} s^{-1} for *N*-dodecyl-D-glucosylamine and appeared to be roughly proportional to $1/K_I$ but there were many exceptions (data not shown). In addition, no generalizations could be made with respect to the type of sugar and substrate analogues that exhibited slow inhibition, even if the K_I-values were of similar magnitude. Examples are **1** (K_I 0.13 μM, slow onset) and *N*-butyl-D-glucosylamine (K_I 1.3 μM rapid onset) with bovine cytosolic β-glucosidase and *N*-tetradecyl-D-glucosylamine (K_I 0.0007 μM, slow onset) and *N*-tetradecyl-1-deoxynojirimycin (K_I 0.0085 μM, rapid onset) with human lysosomal β-glucosidase [37]. Thus, slow inhibition per se cannot be used as a criterion for transition state resemblance, even in combination with K_I-values. Possibly, the dependence of K_I', k_5, and k_6 on inhibitor structure may provide more clearcut evidence, as k_{appr} is determined by both K_I' and the rate constants for the isomerization k_5 and k_6. Their accurate determination met with some difficulties, however, because at low inhibitor concentrations $[I] < K_I$ (to extrapolate for k_6) k_{obs} was too low and a constant rate of substrate hydrolysis was not reached because of substrate depletion. At $[I] > 50$ K_I, the steady state was reached within 10 s, so that $(k_5 + k_6)$ could not be determined with the manual techniques used here.

3. Conclusion

The question raised in the title must clearly be answered by NO. Even for the favourable case of β-glucosidase, A_3 from *Asp. wentii* which is better inhibited by cationic than by basic glycon and substrate analogues K_S/K_I was still ≥ 6 orders of magnitude than the rate acceleration factor k_{cat}/k_{uncat}. With the other enzymes, it might be possible to enhance the inhibitory potency of the gluconamidines by using substituted (e.g. fluorinated) alkylamines with $pK_a < 7$ for their preparation. This would lower the pK_a of amidines, providing a substantial proportion of the unprotonated form under the assay conditions and enabling them to form a hydrogen bond with the catalytic acid. While this might give up to 10^4-fold improvement, it is still far from sufficient to make these compounds 'perfect' transition state mimics. The model shown in Scheme 1 gives little information about bond distance, bond angles and charge distribution at C-1 and the glycosidic oxygen at the moment of bond breaking. The exact requirements cannot be matched by a stable molecule and it may well be that we underestimate the difference in the interaction energies of the enzyme with the 'real' transition state and a stable molecule coming as close to it as the laws of chemistry permit.

4. Experimental

Inhibitors and substrates.—The following compounds were prepared as described: N^1-butyl- (**1**) and N^1-dodecyl-D-gluconamidine (**2**) [3]; *N*-butyl- and *N*-dodecyl-D-glucosylamine [41]; butyl β-D-glucopyranoside [42]; 4-nonylumbelliferyl β-D-glucopyranoside [43]. Dodecyl β-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside were from Sigma, D-gluconamidrazone was a gift from Professor B. Ganem, Ithaca, NY.

Enzymes.—β-Glucosidase A_3 from *Asp. wentii* [13] and bovine lysosomal β-glucosidase [44] were isolated as described. Bovine cytosolic β-glucosidase: commercial β-galactosidase (Sigma G 1875), a crude preparation from bovine liver; the enzyme activity found with β-glucosides as substrate was mainly due to the non-specific cytosolic β-glucosidase/β-galactosidase [3]. The material was used without further purification after inactivation of the contaminating lysosomal β-glucosidase with conduritol B epoxide [3]. β-Glucosidase from almonds and α-glucosidase from yeast were from Sigma (G 4511 and G 7256, respectively).

Inhibition studies.—Concentrations of **1** and **2** were calculated from stock solutions assayed with ninhydrin before and after treatment with dilute NaOH [3]. Stock solutions of D-glucosylamines were freshly prepared in methanol and the required amount added to the substrate cuvette immediately before rate measurements. Control experiments showed that methanol at $\leq 1\%$ had no effect on the rate. Nojirimycin was prepared from its hydrogen sulfite adduct and assayed as described [3].

Enzyme activities were determined fluorimetrically at 25 °C and pH as given in Tables 1 and 2. Fluorescence was recorded continuously, except at pH 7.5 and 8.0 where photodecomposition was minimized by intermittently removing the sample cuvette from the light path for 55 s and measuring for 5 s. Inhibition constants K_I were calculated

from triplicate measurements of the rates in the presence (v_I) and absence of inhibitor (v_0) of concentration I with at least two substrate concentrations S above and below the Michaelis constant K_M, assuming competitive inhibition:

$$K_I = \frac{I}{(v_0/v_I - 1)(1 + S/K_M)}.$$

Where mixed competitive/non-competitive inhibition was indicated by smaller K_I-values calculated from measurements at higher substrate concentrations, the competitive component of the inhibition was evaluated from slopes of Lineweaver–Burk plots with measurements with at least five substrate concentrations $K_t = [1/(slope(I)/slope(0)) - 1]$.

The above formulae are only valid if the total enzyme concentration is much lower than the inhibitor concentration, i.e. $E_t < I_t$. For the bovine cytosolic β-glucosidase and **2** as well as dodecyl glucosylamine, E_t approached $5I_t$, thereby making K_I about 20% larger.

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