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Inhibition of glycosidases by substituted amidines

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Abstract: Five substituted amidines were examined as reversible inhibitors of glycosidases. All compounds exhibit competitive inhibition. The K_i values of the amidines strongly depend on the hydroxylation pattern and on the structure of the aglycon moeity. Best inhibition ($K_i = 10^{-6}$-10^{-7}M) were observed with structures that closely resemble to sugar structure. The pH dependence of a particular potent inhibitor was also examined on α-mannosidase and β-glucosidase. The affinity ($1/K_i$) for α-mannosidase varies with pH indicating that it is the protonated form of the amidine which binds to the enzyme when a enzymic group of $pK_e = 5.2$ is deprotonated.

The design of effective enzyme inhibitors generally relies on the mechanism of the enzyme catalyzed reaction and best results are obtained with structures that closely ressemble the transition state[1]. For glycosidases, the enzymatic mechanism is thought to involve a transient oxocarbenium with flattened chair conformation stabilized by an active site catalytic residue with a complementary charge, identified as a carboxylate in most of glycosidases[2]. As a consequence, protonated amino-sugar derivatives like piperidines, glycosyls amines, pyrrolidines, indolizidines and pyrrolizidines are known to be powerful inhibitors[3]. Their interest relies on the great therapeutic potential in many deseases by altering the glycosylation or catabolism of glycoproteins, or by blocking recognition of specific sugars[4]. Recently, amidine derivatives of sugars whose structure, shape and charge mimick the transient glycosyl cation (Figure 1) have also been proved to be potent and broad spectrum inhibitors of glycosidases[5].

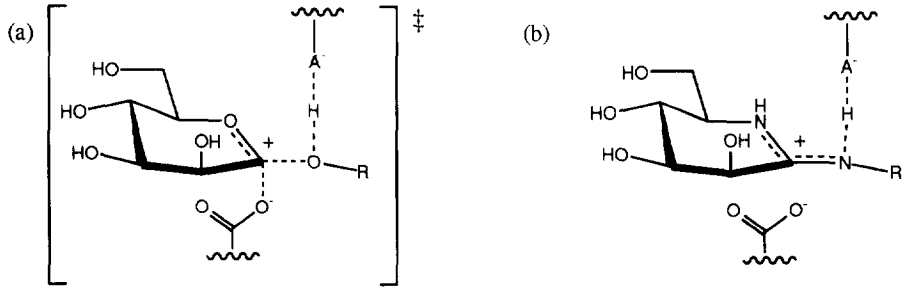
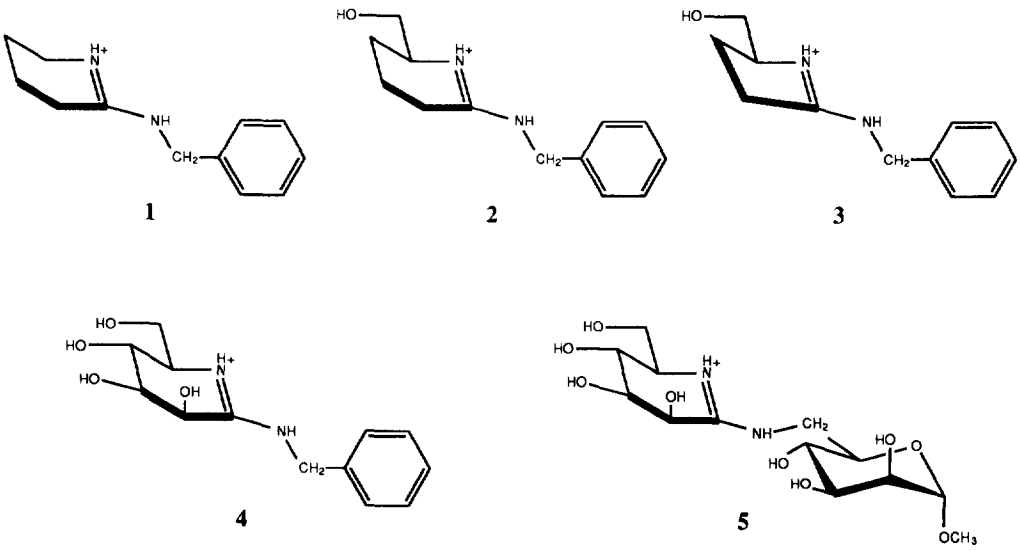


Figure 1 : Analogy between (a) the transition state in the hydrolysis of a β-mannoside and (b) the probable binding mode of a protonated amidine with the active site catalytic residues of a glycosidase.

However, the relative importance of the structure, shape and charge of glycosidase inhibitors are still controversial. Kajimoto *et al*[6] concluded that the charge and half-chair conformation both contribute to strong inhibition and that hydroxyl groups at position 3 and 4 are not essential for the recognition and the binding of inhibitors to glucosidases. Papandreou *et al*[7], by observing a strong inhibition of β-glucosidase with amidine and amidrazone with low pKa concluded that a flattened anomeric configuration is more important. This raises the question whether it is the protonated or the non-protonated inhibitors which bind most tightly the active site. Furthermore, as the rate determining step for the overall hydrolysis proceeds via a transition state involving the departure of the aglycone moiety of the substrate, adding in the inhibitor structural features capable of mimicking the interaction of the aglycone portion of the substrate could be essential for the design of effective transition state inhibitors[8]. Here, cyclic and substituted amidines differing in the cycle size, the hydroxylation pattern or the "aglycon" moeity are tested for their inhibitory activity on several exoglycosidases. We also report a study of the pH-dependence of the binding of the mannoamidine inhibitor **4** to α-mannosidase and β-glucosidase to analyse the importance of the electrostatic effects in the inhibitor binding.



All the amidines **1-5** were obtained from the corresponding lactam which, after suitable protection and treatment with the Lawesson's reagent as reported previously[9] furnished thionolactams. δ- and ω-hydroxymethyllactams yielding **2** and **3** were obtained in three steps from (S)-α-aminoadipic acid and (S)-α-glutamic acid[10]. Protected mannonolactam used as starting material for the synthesis of **4** and **5** was synthesized in six steps according to Fleet *et al* (1989)[11]. Because direct reaction of benzylamine with thionolactams did not furnish the corresponding amidines **1-3**, thioiminoether were synthesized as intermediates[12]. All the amidines are soluble in water as amidinium salts and are stable over months. However, as free bases, these amidines are very sensitive to nucleophiles and start to decompose to lactam at pH > 8[13]. The pK_a values of substituted amidines are very close to those of the corresponding amines[14].

All the synthesized amidines **1-5** are competitive inhibitors of the glycosidases tested (Table 1) at their optimum pH. Amidine **1** which does not contain any hydroxyl group inhibits moderately β-glucosidase and α-mannosidase. This observation confirms the inhibition properties of various non-sugar type amines on glycosidases[15] and demonstrates that electrostatic interactions between the positive charge of the inhibitor and the enzyme is an important factor for binding. Amidine **2**, which possesses an hydroxymethyl group at position 6, binds the α-mannosidase and β-glucosidase by one order of magnitude more strongly than does amidine **1**. However, the inhibition constants K_i are much higher than those predicted by Kajimoto *et al.*[6] from a structure-K_i relation which probably overestimated the binding contribution of the hydroxymethyl group.The increase in binding energy ($\Delta\Delta G \approx 1.4$ kcal/mol) is consistent with the formation of an hydrogen bond between the OH6 and a group of the enzyme active site.

Fully hydroxylated mannoamidines **4** and **5** are among the most potent inhibitors of mannosidases (K_i = 10^{-6}-10^{-7}M). Despite D-manno configuration, **4** and **5** exhibit inhibition properties on β-glucosidase, but no cross-reactivity was observed with galactosidase. As expected from the flattened conformation at the anomeric center, **4** and **5** are good competitive inhibitors against either α- or β-mannosidases. However, the structure of the aglycon moiety seems to influence the selectivity of the inhibition. since the pseudo-dissaccharide **5** shows a better selectivity than **4** or D-mannoamidrazone[7] of two order of magnitude against α-mannosidase versus other glycosidases. On the other hand, the benzyl group which enhances the hydrophobicity of the aglycon moiety increases the affinity to β-glucosidase[15] with no effect on α-mannosidase. The five membered cyclic amidine **3** has no enhanced inhibitory properties with respect to **1** against β-glucosidase despite the presence of an hydroxyl group.

Table 1 Inhibition of glycosidases by amidines based compounds

Compound	K_i (M)[16]			
	α-mannosidase (Jack bean)[a]	β-mannosidase (Snail)[b]	β-glucosidase (Sweet almond)[c]	β-galactosidase (Bovine liver)[d]
1	$1.4\ 10^{-2}$	___	$3.7\ 10^{-3}$	___
2	$1.4\ 10^{-3}$	___	$7.0\ 10^{-4}$	___
3	___	___	$4.0\ 10^{-3}$	___
4	$5.5\ 10^{-7}$	$6.0\ 10^{-6}$	$5.0\ 10^{-6}$ [f]	----[e]
5	$2.6\ 10^{-6}$	$1.2\ 10^{-4}$	$1.0\ 10^{-4}$	----[e]
D-mannoamidrazone[7]	$1.7\ 10^{-7}$	___	$2.0\ 10^{-4}$	$5.7\ 10^{-4}$

[a]p-nitrophenyl α-D-mannopyranoside as substrate, citrate buffer (50mM), pH 4.5.[b]p-nitrophenyl β-D-mannopyranoside as substrate, acetate buffer (50mM), pH 4. [c]p-nitrophenyl β-D-glucopyranoside as substrate, acetate buffer (50mM), pH 5.6. [d]p-nitrophenyl β-D-galactopyranoside as substrate, phosphate buffer (50mM), pH 7. [e]No inhibition at 1 mM. [f]Slow binding inhibition was observed and inhibition constant including the slow-onset component (K_i^*)[17] was determined with 4-methylumbelliferyl-β-D-glucopyranoside.

The effect of pH on the binding of the inhibitor was estimated by measuring the inhibition constants at different pH values ranging from 3 to 6. Jack bean α-mannosidase presents a pH dependence of the inhibition by all the amidines whereas inhibition of sweat almond β-glucosidase is pH independent. The pH dependence for the binding ($1/K_i$) of mannoamidine **4** to α-mannosidase is illustrated in Figure 2 showing that inhibition increases as the pH increases. As amidine **4** (pK_a = 10.0) was fully protonated over the pH range, the pH profile shows that the inhibitor binding depends on the protonation state of a group with a pK_e of 5.2 at the enzyme binding site.

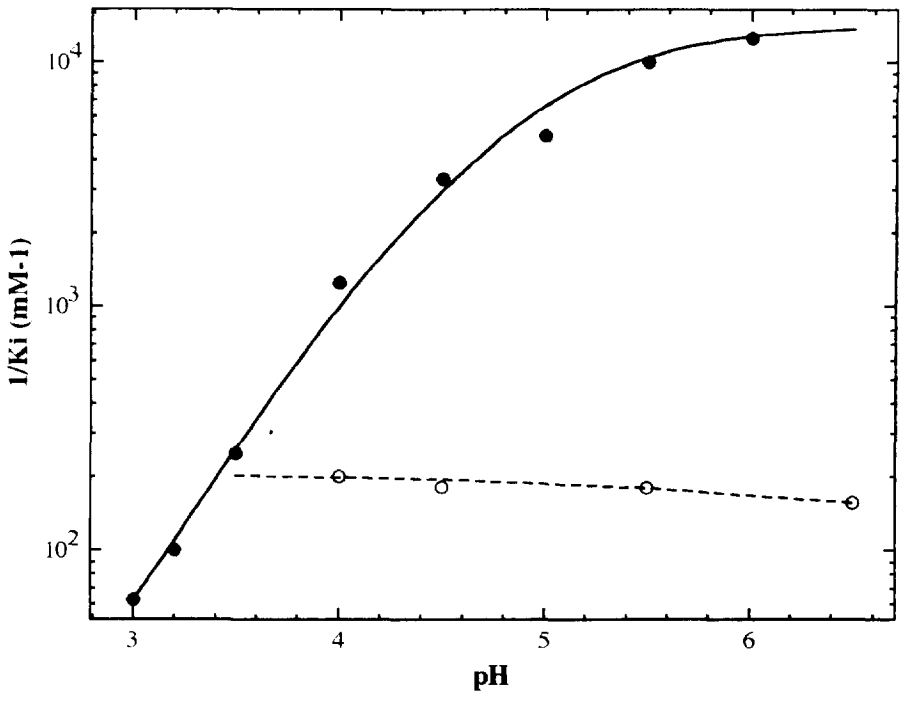


Figure 2. pH dependence[19] for the binding of amidine **4** to α-mannosidase (●) and β-glucosidase (o). The data were fit to eq: $1/K_i = (1/Ki_{low} + 1/Ki_{high} \cdot 10^{pH-pK\,e}) / (1 + 10^{pH-pK\,e})$ where K_e is the ionization constant for a group of the enzyme and Ki_{low} and Ki_{high} are the dissociation constants of **4** from the protonated and the deprotonated enzyme. The solid line is the theoretical curve with pK_e = 5.2, $Ki_{low} = 7\ 10^{-6}$ M and $Ki_{high} = 6.6\ 10^{-8}$ M.

A detailed consideration of the likely transition states for glycosidase catalysis suggests a reason for this behaviour. Proton donation to the leaving group is commonly invoked as the first step in glycosidase catalysis, and such proton donation is believed to involve a catalytic group in the enzyme active site that would be partly deprotonated at the transition state (figure 1). Therefore, if the protonated amidines do indeed resemble the

glycosyl moiety at enzymic transition state, these transition-state analogues should bind more tightly the deprotonated enzyme, the catalytic group being partly deprotonated at this stage. The effect will be reinforced by simple electrostatic: binding of a positively charged inhibitor is enhanced if the active site decreases in charge by 1 unit[18]. With α-mannosidase, maximal inhibition is therefore observed above the pH optimum of the enzyme. In principle, these data could also be interpreted as binding of deprotonated inhibitor to protonated enzyme as it has been suggested for the binding of deoxynojirimycin to β-glucosidase[15]. However, this would mean that binding of mannoamidine to enzyme would induce a 5 units decrease (10.0 to 5.1) of the amidine pK_a which would occur at the expense of binding energy. We can conclude that α-mannosidase is better inhibited by the inhibitor cation benzyl-D-mannoamidinium salt **4**. Taking into account the difference in the binding constant to the protonated ($1/Ki_{low} = 1.4\ 10^5\ M^{-1}$) and the deprotonated enzyme ($1/Ki_{high} = 1.5\ 10^7\ M^{-1}$), one can conclude that the enzyme deprotonation results in an increase of the binding energy by 2.8 kcal/mol mainly by electrostatic effect. With β-glucosidase, we observed a pH-independent inhibition by the benzyl-D-mannoamidine **4** as already shown with other glycoamidinium salts[7]. β-glucosidase belongs to a class of enzymes where the inhibiting species is the unprotonated form of the inhibitor; permanently cationic inhibitors are bound only to the same extent as their neutral counterparts[2]. A possible explanation for the behaviour of this class of enzymes could be the presence of a cationic acid (e.g. protonated histidine) at the catalytic site. A permanently cationic inhibitor would be attracted by the active site carboxylate but it would be at the same time repelled by the cationic acid group.

In summary, fully hydroxylated amidines by developing partial positive charge as well as a flattened chair conformation characteristic of the glycosyl cation are potent glycosidase inhibitors. However, for enzyme inhibition, the importance of the positive charge in the inhibitor strongly depends on the enzyme source. The presence of an aglycon portion in the amidine inhibitors does not significantly improve the inhibition but induces an effective selectivity.

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