Bioorganic & Medicinal Chemistry Letters, Vol.1, No.12, pp. 667-672, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

The Interaction of Anhydroalditols with Sweet-Almond β-Glucosidase and *Escherichia coli* β-Galactosidase: Implications for the Design of Potent Glycosidase Inhibitors

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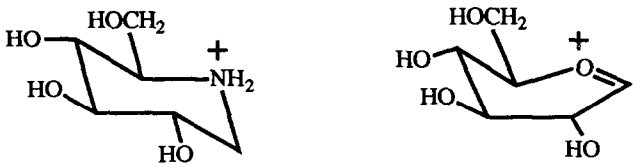
(*Received* 12 *June* 1991)

Abstract: A range of 1,4- and 1,5-anhydroalditols have been synthesized and assessed for their ability to inhibit glycosidases. Observed inhibition indicates that aglycone-enzyme interactions contribute significantly to both the affinity and the stereoselectivity of substrate binding. Such interactions may also contribute to enzyme-transition state interactions. Implications for the design of potent glycosidase inhibitors are discussed.

The scientific literature over the past few years contains a plethora of examples of potent iminoalditol glycosidase inhibitors related to 1-deoxynojirimycin (5-amino-1,5-dideoxy-D-glucopyranose)(see ref.1, for example). It was initially considered that such compounds acted as transition state analogues due the charge similarity of the protonated form of the inhibitor with the oxocarbonium ion-like transition state for enzymatic glycoside hydrolysis (Fig.1)[2]. However, as discussed in the literature[3,4], a number of features of the inhibitory properties reported for this class of compounds are inconsistent with their designation as transition state analogues.



(a) (b)

Figure 1: (a) Protonated form of 1-deoxynojirimycin; (b) D-glucopyranosyl oxocarbonium ion.

The design of effective enzyme inhibitors relies on an indepth knowledge of the mechanism of the enzyme catalyzed reaction to be inhibited[5]. Whilst there are numerous examples of studies on enzyme catalyzed glycoside hydrolysis, mechanistic uncertainties are still apparent[5]. Kinetic studies on sweet-almond β-glucosidase by Dale *et al* [6], which is further supported by other literature data[5], show that this enzyme catalyzes the hydrolysis of substituted phenyl β-D-glucopyranosides by a multi-step process via a covalent α-D-glucopyranosyl-enzyme intermediate. For all but exceptionally reactive phenyl β-D-glucosides (such as the 2,4-dinitrophenyl compound), the rate determining step for overall hydrolyisis proceeds via a transition state involving departure of the aglycone moiety of the substrate[6,7] (Fig. 2).

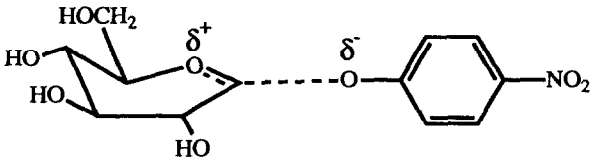


Figure 2: Rate-determining transition state for β-glucosidase catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside.

In view of this transition state structure, it would seem reasonable to suggest that transition state analogue inhibitors of sweet-almond β-glucosidase should contain, in addition to a carbohydrate-based ring, structural features capable of mimicking the interaction of the aglycone portion of the substrate (the phenyl ring in this case) with the enzyme. The importance or otherwise of aglycone-enzyme interactions in the ground state or transition state has not previously been investigated. For reasons of synthetic expediency, we chose to investigate the former by studying the interaction of simple 1,5-anhydroalditols, such as 1,5-anhydro-D-glucitol (Table 1, *Gluco* isomer), with sweet-almond β-glucosidase and *E. coli* β-galactosidase.

1,5-anhydroalditols were synthesized using literature procedures or modifications thereof. Reduction of *per*-acetyl glycosyl halides with tributyltin hydride / AIBN[9] or TMS-triflate / Et_3SiH reduction[10] of appropriately protected methyl glycosides gave 1,5-anhydroalditols in moderate to good yield[11]. All compounds were judged to be single stereo- and regioisomers by proton decoupled ^{13}C NMR spectroscopy. The inhibitory effect of these compounds on sweet-almond β-glucosidase (isozyme A, see ref. 12) and *E. coli* β-galactosidase were assessed as described previously[12] and data obtained are summarized in Table 1. Where investigated, inhibition proved to be purely competitive with respect to the *p*-nitrophenyl glycoside used as a substrate.

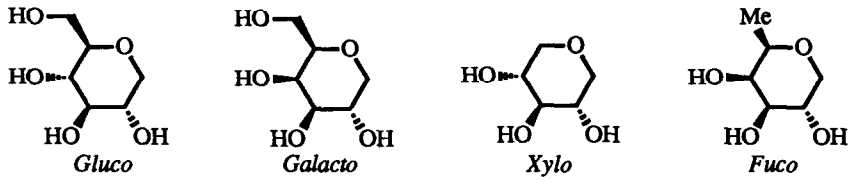
The data analysis used in this report uses the arguments developed by Bartlett and Marlowe[13]. For a range of substrate analogue inhibitors a direct linear correlation between the K_i value for an inhibitor and the Michaelis constant, K_m, for the corresponding substrate would be expected[14]. This requires that K_m equates to the substrate binding constant, K_s, which, although not always the case, is consistent with literature data used in the current study[14]. Additionally, for transition state analogue inhibitors a direct linear correlation is found between K_i and K_m/k_{cat} for the corresponding substrate[13,14]. With respect to the data presentation in this report, such correlations would require equivalent K_i/K_m values for good substrate analogue inhibitors and equivalent $K_i/(K_m/k_{cat})$ values for good transition state analogue inhibitors.

From the data presented in Table 1 it is apparent that *p*-nitrophenyl glycosides bind more strongly to the enzyme than 1,5-anhydroalditols of the same stereochemistry[14], *i.e.* K_i/K_m (Table 1) are in all cases greater than one. As one would expect, 1,5-anhydroalditols do not act as good transition state analogue inhibitors of either of the enzymes investigated in this study; $K_i/(K_m/k_{cat})$ values vary over several orders of magnitude (Table 1). It would appear that the *p*-nitrophenyl ring of the glycoside substrates contributes favourably to substrate binding. On the basis of the available data it is not possible to distinguish between a favorable enthlapic interaction between enzyme and substrate aglycone or tighter binding arising from entropically favourable displacement of water molecules from the enzyme active site by the phenyl ring of the substrate. It is also clear from Table 1 that both of the enzymes tested in this study are less able to discriminate between 1,5-anhydroalditols than *p*-nitrophenyl-β-D-glycopyranosides (for similar ability to discriminate, the K_i/K_m values in Table 1 should be equivalent). This suggests that not only does the enzyme-aglycone interaction contribute to the affinity of substrate binding but also

contributes in some manner to the stereoselectivity of this binding. It would seem reasonable to expect such interactions to also play a role in enzyme-transition state recognition processes and consequently contribute to the selectivity of enzyme caytalyzed glycoside hydrolysis.

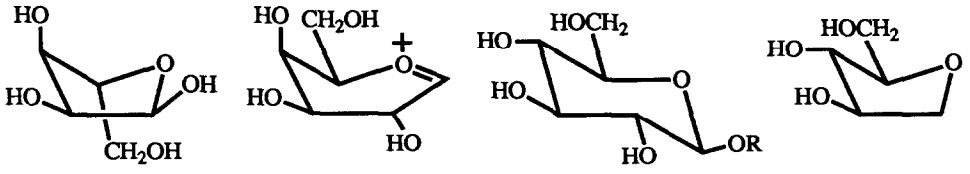
Table 1: Inhibition of Sweet-Almond β-Glucosidase and *E. coli* β-Galactosidase by 1,5-Anhydroalditols. Comparison of K_i values for 1,5-Anhydroalditols with K_m and K_m/k_{cat} Values for the Corresponding *p*-nitrophenyl β-D-glycopyranosides.

		β-Glucosidase			
Isomer	K_i(mM)	K_m(mM)[a]	K_i/K_m	K_m/k_{cat}[b]	$K_i/(K_m/k_{cat})$
Gluco	60	2.5	24	0.019	3160
Galacto	140	15.7	8.9	0.34	410
Xylo	39	3.1	12.6	1.0	39
		β-Galactosidase			
Isomer	K_i(mM)	K_m(mM)[a]	K_i/K_m	K_m/k_{cat}[b]	$K_i/(K_m/k_{cat})$
Gluco	71	32.6	2.2	0.21	[c]330
Galacto	7.4	0.048	154	0.19×10^{-7}	39×10^{7}
Xylo	85	15.2	15.6	1.0	[c]85
Fuco	42	35.9	1.2	6.7×10^{-5}	6.3×10^{5}



[a] k_{cat} and K_m values were obtained from references 3 and 15 respectively[16]; [b] K_m/k_{cat} values are normalized with respect to *p*-nitrophenyl β-D-xylopyranoside as 1.0; [c] upper limit[16].

Huber and Brockbank[17] have suggested that the strong inhibition of *E. coli* β-galactosidase by L-ribose may be due to the potential geometrical similarity of the β-furanose form of the sugar to the oxocarbonium ion-like transition state for galactoside hydrolysis [Fig. 3, structures (a) and (b) respectively]. It is possible that substrate destabilization[18] resulting from interaction of C-1 of the substrate with the enzyme may be relieved in the transition state by adoption of a half-chair conformation [Fig. 3, (b)]. Such unfavourable interactions may not be encountered by some glycofuranoses bound to glycosidases since pentofuranoses of appropriate stereochemistry can be viewed as pyranoses in which C-1 has been excised and the ring oxygen atom joined to C-2.



(a) (b) (c) (d)

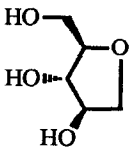
Figure 3: (a) β-L-ribofuranose; (b) D-galactopyranosyl oxocarbonium ion; (c) β-D-glucopyranoside; (d) 1,4-anhydro-D-arabinitol.

Huber and Brockbank[17] considered β-L-ribofuranose to show structural similarity to the galactopyranosyl oxocarbonium ion-like transition state for D-galactpyranoside hydrolysis, despite the incorrect stereochemistry at C-5 of the ribofuranose. A similar argument can be used, perhaps more convincingly, for analogy between β-D-glucopyranosides [Fig. 3, (c)] and 1,4-anhydro-D-arabinitol [Fig. 3, (d)].

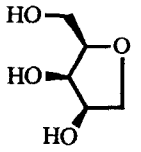
With a view to investigating this point, a number of 1,4-anhydroalditols, such as 1,4-anhydro-D-arabinitol (ψ-*Gluco* isomer[19], Table 2), were synthesized using similar methods to those used to prepare the 1,5-anhydroalditols. 1,4-anhydro-D-threitol which was prepared by the acid catalyzed dehydration of D-threitol as described for the formation of 1,4-anhydroerythritol[20]. The inhibitory properties of the synthetic 1,4-anhydroalditols are summarized in Table 2.

Table 2: Inhibition of Sweet Almond β-Glucosidase and *E. coli* β-Galactosidase by 1,4-Anhydroalditols. Comparison of K_i values for 1,4-Anhydro-D-alditols with K_m and K_m/k_{cat} values for the Corresponding *p*-nitrophenyl β-D-glycopyranosides.

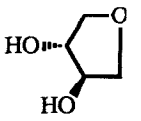
			β-Glucosidase		
Isomer[19]	K_i(mM)	K_m(mM)[a]	K_i/K_m	K_m/k_{cat}[b]	$K_i/(K_m/k_{cat})$
ψ-*Gluco*	36.2	2.5	14.5	0.019	1900
ψ-*Galacto*	14.8	15.7	0.9	0.34	44
ψ-*Xylo*	390	3.1	126	1.0	390
			β-Galactosidase		
Isomer[17]	K_i(mM)	K_m(mM)[a]	K_i/K_m	K_m/k_{cat}[b]	$K_i/(K_m/k_{cat})$
ψ-*Gluco*	51	32.6	1.6	0.21	[c]240
ψ-*Galacto*	1.9	0.048	39.6	1.9×10^{-8}	1.0×10^{8}
ψ-*Xylo*	77	15.2	5.1	1.0	[c]77



ψ-*Gluco*



ψ-*Galacto*



ψ-*Xylo*

[a] k_{cat} and K_m values were obtained from references 3 and 15 respectively[16]; [b] K_m/k_{cat} values are normalized with respect to *p*-nitrophenyl β-D-xylopyranoside as 1.0. [c] upper limit[16]

From the data presented in Table 2 there is clearly no correlation between K_i for 1,4-anhydroalditols and K_m/k_{cat} for the corresponding *p*-nitrophenyl glycosides for either of the enzymes investigated. This is to be expected if aglycone-enzyme interactions play a key role in transition state recognition by glycosidases[21]. In addition, there is no obvious correlation between K_i for the 1,4-anhydroalditols and K_m for the corresponding *p*-nitrophenyl β-D-glycopyranosides indicating that not only do these compounds not act as good transition state analogues, but that they also act as poor substrate analogue inhibitors for both sweet-almond β-glucosidase and *E. coli* β-galactosidase. It should be noted, however, that for the ψ-*Galacto* isomer with sweet-almond β-glucosidase, K_i/K_m is less than one indicating tighter binding of the inhibitor than the corresponding substrate.

This is in contrast to the situation recorded for the *Galacto* isomer with the same enzyme (K_i/K_m=8.9) which may suggest a difference in the mode of binding of these two inhibitors.

The design of potent glycosidase inhibitors has routinely relied on a carbohydrate-based substructure of some form, capable only of interacting with the glycone binding site on the enzyme. However, data presented in this report indicates that interactions between substrates / inhibitors and the aglycone binding site on the enzyme contribute significantly to both the affinity and stereoselectivity of substrate binding and may also contribute to enzyme-transition state recognition processes. If this is in fact the case, the lack of aglycone structure in simple iminoalditols may go some way to explain the lack of stereoselectivity in the inhibitory properties of these compounds and their inability to act as true transition state analogue inhibitors[4].

The interaction of inhibitors with the aglycone binding site of glycosidases is a neglected area of study, although there are a number of reports in the literature of hydrophobic molecules, some of which do not contain a sugar ring, which act as potent glycosidase inhibitors[22]. For instance, we have recently shown[12] that ω-N-benzoyl histamine inhibits sweet-almond β-glucosidase with a pH-independent K_i=0.11μM. This inhibition is at least eighty-fold stronger than the interaction of 1-deoxynojirimycin (pH-independent K_i=9.5μM)[26] with the same enzyme.

On the basis of literature reports[5] and data presented in this study it would seem reasonable to suggest that potent glycosidase inhibitors should contain structural features capable of a) interacting with the glycone binding site, b) of interacting with the catalytic acid / base at the enzyme active site[27], and c) of interacting with the aglycone binding site of the enzyme.

Acknowledgements: This work was supported by the U.K. Science and Engineering Research Council and ICI Agrochemicals in the form of a CASE Award studentship to R.A.F..

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7. The possibility of a preassociation mechanism (see, for example, ref. 5) involving an exploded transition state[8] containing both departing aglycone and incoming nucleophile, in this case a carboxylate residue from the enzyme active site[5], cannot be excluded.

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14. The assumption that $K_m = K_s$ is consistent with data for both sweet-almond β-glucosidase and *E. coli* β-galactosidase with *p*-nitrophenyl-β-D-gluco- and galactopyranosides as substrates respectively[6,15].

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16. For *E. coli* β-galactosidase with very poor substrates such as *p*-nitrophenyl β-D-gluco- and xylopyranosides, K_i values for the 'substrate' are substituted for K_m values and upper limiting values of k_{cat} have been used (ref. 15), thus giving rise to upper limits for $K_i/(K_m/k_{cat})$.

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19. In Table 2, ψ-*Gluco* (pseudo-*Gluco*) refers to 1,4-anhydro-D-arabinitol, ψ-*Galacto* to 1,4-anhydro-D-lyxitol, and ψ-*Xylo* to 1,4-anhydro-D-threitol, the ψ-terminology indicating the hexopyranoside or pentopyranoside to which the anhydropentitol or anhydrotetritol, respectively, bears a direct stereochemical relationship.

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21. To act as true transition state analogue glycosidase inhibitors the anhydroalditols would need to acquire at least a partial positive charge in the enzyme-inhibitor complex which is unlikely to occur.

22. For example, ephedrine[3], N-Benzyl-D-glucosylamine[23], 4-phenylimidazole[24], and a hydrophobic gluconhydroximinolactone[25] have all been shown to act as potent inhibitors of sweet almond β-glucosidase.

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26. pH-Independent K_i value calculauted using K_i=18μM at pH 6.75 and inhibitor pK_a=6.7 assuming only the basic form of the inhibtor is inhibitory[3].

27. For example by electrostatic interaction following protonation of a basic inhibitor by the enzyme.