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CONDURITOL AZIRIDINE: A NEW MECHANISM-BASED GLUCOSIDASE INACTIVATOR

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A new mechanism-based glucosidase inactivator, conduritol aziridine (1,2-dideoxy-1,2-epimino-*myo*-inositol), has been synthesised from *myo*-inositol. This aziridine inactivates both the β-glucosidase from *Alcaligenes faecalis* and the α-glucosidase from yeast according to the expected pseudo-first order kinetics. Inactivation constants measured are Ki = 3.0mM, ki = 0.077 min^{-1} for the β-glucosidase, and Ki = 9.5mM, ki = 0.39 min^{-1} for the α-glucosidase. Evidence for irreversible inactivation is provided by the lack of reactivation upon dilution of inactivated enzyme into buffer containing substrate.

Considerable interest has been shown, particularly of late, in the design and synthesis of specific mechanism-based glycosidase inhibitors. Such inhibitors are of interest both in the study of the enzymes themselves (1) and in the study of mechanisms of glycoprotein processing (2). They also have potential as therapeutic agents in the treatment of diabetes and obesity by direct interference with starch digestion (3), as well as in diseases such as AIDS through interference with the normal processing of the viral glycoprotein (4).

Glycosidase inhibitors of both the non-covalent and covalent type are known. Some of the most effective of the non-covalent inhibitors include the naturally-occurring carbohydrates acarbose and nojirimycin, as well as several alkaloids such as castanospermine and swainsonine (1,3). These are all nitrogen-containing sugars, or mimics thereof, which bind tightly at the enzyme active site, likely by virtue of favourable electrostatic interactions between the enzyme and the inhibitor developed upon protonation of the inhibitor nitrogen. An enormous effort has been directed recently at the

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chemical synthesis of these compounds and analogues thereof in a search for better inhibitors.

A number of covalent inhibitors have been synthesised and tested, ranging from simple affinity labels such as N-bromoacetyl-β-D-glucosylamine (5) and glucosyl isothiocyanate (6) to a number of mechanism based inactivators. These include conduritol epoxides (7), glycosylmethyl triazenes (8), 2-deoxy-2-fluoro-glycosides (9), 1',1'-difluoroalkyl glycosides (10) and recently an aziridine derivative based upon nojirimycin (11). The first of these, the conduritol epoxides, bind at the active site by virtue of their resemblance to the natural substrate and are then protonated by the enzymic acid-catalyst, rendering the epoxide highly reactive towards nucleophilic attack. Opening of the epoxide by attack of an enzyme nucleophile (a carboxylate which functions as the nucleophile in the normal reaction has been labelled in several cases (1)) results in covalent derivatisation of the enzyme, thus inactivation.

In this paper we describe the synthesis and testing of an inhibitor, conduritol aziridine (structure shown in Figure 1), which combines the crucial element of the best non-covalent inhibitors, the protonatable nitrogen, with the reactivity of the conduritol epoxide in the hope of creating a highly reactive inactivator which interacts very strongly with the enzyme prior to covalent derivatisation.

MATERIALS AND METHODS

Buffer chemicals and substrates were all obtained from Sigma Chemical Company, as was the α-glucosidase (Type III from yeast). The β-glucosidase is an enzyme isolated from *Alcaligenes faecalis* (12) and since cloned into *Escherichia coli* (13). This enzyme was isolated essentially as described previously (12).

The synthesis of conduritol aziridine will be described in detail elsewhere. In brief, selective displacement of the axial mesylate of 2,3-di-O-mesyl-1,4,5,6-tetra-O-benzyl-*myo*-inositol by azide yielded a mono-azido, mono-mesylate derivative which could be transformed into the desired product, 1,2-dideoxy-1,2-epimino *myo*-inositol (conduritol aziridine) by catalytic hydrogenation and cyclisation. The structure of the final product was confirmed by 300 MHz ^{1}H-NMR as follows: δ 3.88 (m, 1 H, H-3), 3.68 (m, 1 H, H-5), 3.20 (m, 2 H, H-4,6), 2.61 (dd, 1 H, J 6.2, 3.3 Hz, H-2), 2.34 (d, 1 H, J 6.2 Hz, H-1).

Inactivation experiments were performed by incubating the enzymes in 50mM sodium phosphate buffer, pH 6.8 in the presence of different concentrations of the aziridine. Aliquots (10μL) were removed at appropriate time intervals and assayed for residual enzyme activity by dilution into a large volume (1.0mL) of substrate p-nitrophenyl glucoside (1.1mM of the β-glucoside for β-glucosidase and 2.8mM of the α-glucoside for α-glucosidase). This effectively halts the inactivation both by diluting the inactivator and by

providing high concentrations of a competitive ligand, the substrate. Activity was determined by continuous monitoring of nitrophenolate release through the increase in absorbance at 400nm. All assays on th β-glucosidase were performed at 37°C while those on the α-glucosidase were performed at 25°C.

RESULTS AND DISCUSSION

Successful synthesis of the conduritol aziridine was evidenced by the NMR data quoted. Resonances observed which are characteristic of aziridines are the doublet at δ2.34 ($J_{1,2}$ = 6.2Hz) and the double doublet at δ2.61 ($J_{1,2}$ = 6.2, $J_{2,3}$ = 3.3Hz). This 6.2 Hz coupling constant, and the absence of observable coupling between H-1 and H-6 are typical of aziridines fused to six-membered rings (14) .

This aziridine molecule has an interesting symmetry, identical to that exhibited by the conduritol epoxide, such that the inhibitor can be expected to bind to, and inactivate both α- and β-glucosidases since a simple inversion of the structure converts the inhibitor from a derivative with the aziridine nitrogen pointing down, and all the hydroxyl groups equatorial, to one with the aziridine up, and all the hydroxyl groups equatorial.

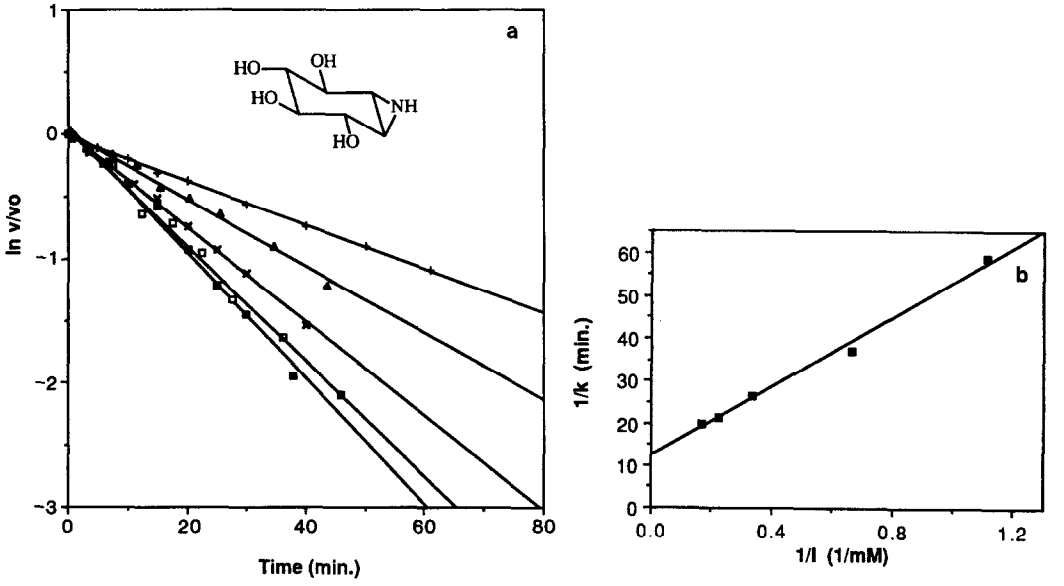

Figure 1. Inactivation of *A. faecalis* β-glucosidase by conduritol aziridine. (a) Plot of ln residual activity ratio versus time; experimental conditions as described in the text. Concentrations of conduritol aziridine employed were: (■) 6 mM; (□) 4.5 mM; (X) 3 mM; (▲) 1.5 mM; (+) 0.9 mM.
(b) Replot of first order rate constants from (a).

The aziridine proved to be an effective inactivator of both the β-glucosidase and the α-glucosidase, showing saturable first order kinetics of inactivation as expected for an inactivator which binds reversibly to the enzyme prior to covalent bond formation. Inactivation data for the β-glucosidase are presented in Figure 1 in the form of a logarithmic plot of residual activity ratio versus time. All plots are cleanly linear, as required for first order kinetics and their slopes provide the pseudo first order rate constants for inactivation at each inhibitor concentration. A replot of the reciprocal of these rate constants versus reciprocal inactivator concentration is a straight line yielding a value for the equilibrium constant for initial binding of Ki = 3.0 mM and an inactivation rate constant, ki = 0.077 min^{-1}. Thus, at saturating concentrations of the inactivator, the inactivation process has a half life of 9 minutes. Protection against inactivation was provided by the competitive inhibitor, β-glucosyl benzene since addition of 7mM glucosyl benzene (Ki = 3.4 mM) to an inactivation mixture containing 4.5 mM conduritol aziridine reduced the apparent inactivation rate from 0.05 min^{-1} to 0.03 min^{-1}. This provides substantive evidence that the inactivation process observed is indeed occurring at the active site of the enzyme.

Good inactivation of the yeast α-glucosidase was also measured. Inactivation data are presented in similar fashion in Figure 2 and once again,

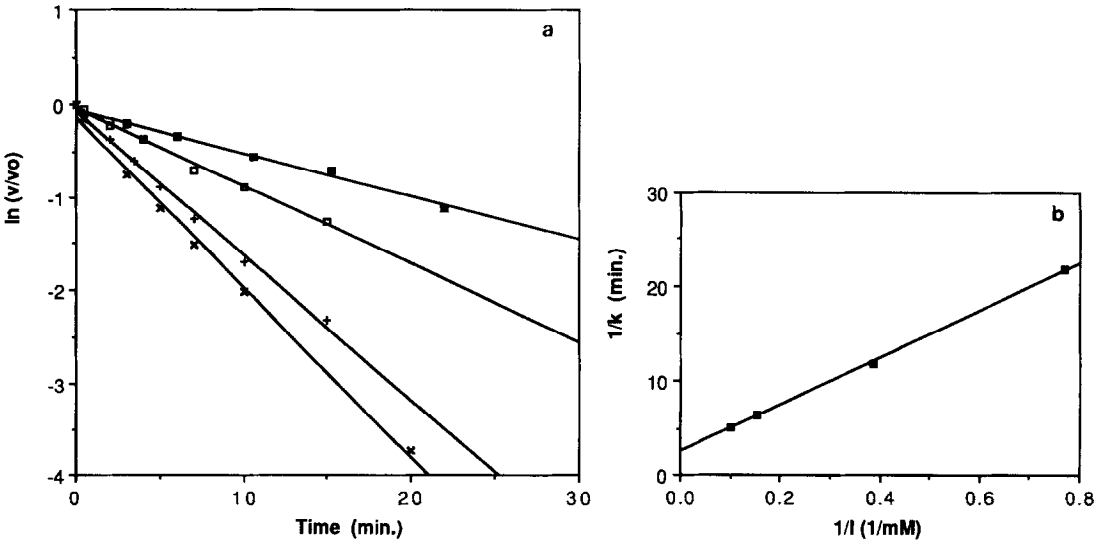

Figure 2. Inactivation of yeast α-glucosidase by conduritol aziridine.
(a) Plot of ln residual activity ratio versus time; experimental conditions as described in the text. Concentrations of conduritol aziridine employed were: (X) 9.9 mM; (+) 6.6 mM; (□) 2.6 mM; (■) 1.3 mM.
(b) Replot of first order rate constants from (a).

first order kinetic behaviour is observed, the replot of the pseudo-first order rate constants yielding values of Ki = 9.5 mM and ki = 0.39 min^{-1}. Thus, the aziridine is slightly more effective with the α-glucosidase, since at saturating concentrations, the inactivation process has a half life of only 1.7 minutes. Further, the equilibrium constant compares very favourably with the Ki of 30mM for methyl α-D-glucopyranoside, and with *myo*-inositol itself, which did not provide measurable inhibition, even at concentrations of 30 mM.

In summary, this new mechanism-based inactivator is very effective with both the β-glucosidase tested and the α-glucosidase. Initial binding is quite reasonably tight, considering the fact that the inhibitor is truly only a xylose analogue. An inhibitor which is more specific for anomeric configuration and considerably more reactive could be generated by the introduction of a hydroxymethyl group at C-5, such that it better resembles the natural glucoside substrate. Such work is in progress.

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