Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 175-180

# KINETIC STUDIES ON CYCLOPHELLITOL ANALOGUES— MECHANISM-BASED INACTIVATORS

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Received June 22, 1995

**SUMMARY:** The (1*R*,6*S*)- and (1*R*,2*S*,6*S*)-diastereoisomers of cyclophellitol were found to be effective irreversible inactivators of α-D-glucosidase and α-D-mannosidase, respectively. The (1*R*,6*S*)-diastereoisomer inactivates brewers yeast α-D-glucosidase according to pseudo-first order kinetics with inactivation constants of $K_i$ = 26.9 μM, $k_i$ = 0.401 $min^{-1}$ while the (1*R*,2*S*,6*S*)-diastereoisomer inactivates jack beans α-D-mannosidase in a similar manner with $K_i$ = 120 μM, $k_i$ = 2.85 $min^{-1}$. The irreversibility of these compounds was evidenced by the lack of reactivation upon dialysis of the inactivated enzyme. 

Synthesis and evaluation of glycosidase inhibitors is the subject of considerable current research (1,2). Glycosidases are essential for the normal growth and development of all organisms, and they are involved in many areas of metabolism and turnover such as breakdown of dietary carbohydrates (3), glycoprotein processing (4), and catabolism of polysaccharides and glycoconjugates (5). Inhibition of these enzymes has promising therapeutic applications since they may be used as antihyperglycemic agents for the treatment of diabetes (6), as inhibitors of tumor metastasis by changing the saccharide structure on tumor cell surface (7), and as drugs to prevent obesity (8). An additional stimulus of great impact on the use of glycosidase inhibitors came from recent studies with the human immunodeficiency virus (HIV) which has a heavily glycosylated protein coat (9).

Structural information for glycosidases is limited except for those from the X-ray crystallographic analysis of some polysaccharide degrading enzymes, and no structural information is available for glycosidases that hydrolyse monosaccharide glycosides. An alternative approach for the identification of important active-site residues in glycosidases involves the use of mechanism-based inactivator or affinity label (2). The crucial distinction between mechanism-based inactivators and other classes of enzyme inhibitors is the requirement for the catalytic action of the target enzyme to unmask the reactive functionality

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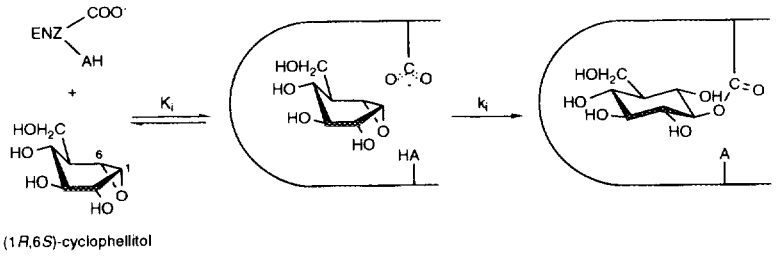


FIGURE 1. Proposed mechansim of irreversible binding of (1*R*,6*S*)-cyclophellitol towards α-D-glucosidase.

of the inactivators. Examples of mechanism-based inactivators include cyclophellitol (10), conduritol epoxide (1) and aziridine (11), glycosylmethyl triazenes (12), 2-deoxy-2-fluoro-glycosides (13), and the dihaloalkyl glycosides (14).

Cyclophellitol was isolated from the culture filtrates of *Phellinus sp.* in 1989 (15) and was identified as a highly specific and irreversible mechanism-based inactivator of β-glucosidase (10). As part of our programme to study the mode of action on glycosidases, the diastereoisomers of cyclophellitol were synthesized (16) and their inhibitory activities had been determined (17). The unnatural diastereoisomers having the α-D-*gluco*-, β-D-*manno*-, and α-D-*manno*-configurations have been characterized as specific inhibitors of α-D-glucosidase from brewers yeast, β-D-mannosidase from *Asp. oryzae*, and α-D-mannosidase from jack beans, respectively (17). The proposed reaction scheme for the inactivation of glycosidases by this class of pseudo-epoxy sugars is illustrated in Figure 1. Based on the knowledge of inactivation of conduritol epoxides towards glycosidases (1), the inactivation of cyclophellitol analogues towards glycosidases should proceed similarly by the initial formation of a noncovalent enzyme-inhibitor complex with an equilibrium constant for initial binding, $K_i$. An acidic group AH will transfer a proton to the oxirane of the inactivator and a carboxylate group will then form an ester bond with the activated oxirane, giving a covalent enzyme-inhibitor complex with rate constant, $k_i$. In this manuscript, we further disclose our findings on the kinetics of the inhibitory action of the (1*R*,6*S*)- and the (1*R*,2*S*,6*S*)-diastereoisomers of cyclophellitol.

## MATERIALS AND METHODS

Chemicals: The (1*R*,6*S*)- and (1*R*,2*S*,6*S*)-diastereoisomers of cyclophellitol were prepared as reported by us (16). *p*-Nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-manno-pyranoside, α-D-glucosidase (EC 3.2.1.20) from brewers yeast and α-D-mannosidase (EC 3.2.1.24) from jack beans were purchased from Sigma Chemical Co., St. Louis, U. S. A. and used without further purification. All reagents were of the highest grade available and used as purchased. pH values were determined at 25 °C.

Kinetic Analysis: Inactivation experiments were performed by incubating the enzyme in the buffer in the presence of inactivators. Aliquots (10 μL) were removed at appropriate time intervals and assayed for residual enzyme activity by dilution into a large volume (1.49 mL)

of saturating concentrations of the corresponding *p*-nitrophenyl glycoside substrate in the same buffer system. This effectively stops the inactivation both by diluting the inactivator enormously, and by providing high concentrations of a competitive ligand, the substrate. Activity was determined by continuous monitoring of *p*-nitrophenolate released through the increase in absorbance at 400 nm. Buffer systems, temperatures and substrates employed for the inactivation experiments for each enzyme were as follows: Brewers yeast α-D-glucosidase, 50 mM sodium phosphate buffer (pH = 6.8), 25 °C, *p*-Nitrophenyl-α-D-glucopyranoside; jack beans α-D-mannosidase, 50 mM sodium phosphate buffer (pH = 6.0), 25 °C, *p*-nitrophenyl-α-D-mannopyranoside.

Glycosidase Activity after Dialysis: Various concentrations of inactivators (0.5 mL) were mixed with the corresponding buffer (100 mM, 0.5 mL) and enzyme (2.5 U/mL for both α-D-glucosidase and α-D-mannosidase, 1 mL) and the mixture was incubated for 11 hours at 4 °C. Half of the volume was dialysed against the same buffer (10 mM), the dialysed sample; and the rest was allowed to stand for 11 hours at 4 °C, the control sample. For α-D-mannosidase, the dialysed and the control sample had to be diluted ten-fold prior to performing the assay. The glycosidase activity was then detected as described previously (17).

## RESULTS AND DISCUSSION

The (1*R*,6*S*)-diastereoisomer inactivates Brewers yeast α-D-glucosidase according to pseudo-first order kinetics as shown in Figure 2 in the form of semi-logarithmic plot of residual activity versus time. All plots are cleanly linear, as required for first order kinetics and their slopes gave the apparent first order rate constants, $k_{app}$, for inactivation at each inhibitor concentration. The apparent first-order rate constant, $k_{app}$, is related to the inhibitor concentration [I] by the following equation $\frac{1}{k_{app}} = \frac{K_i}{k_i}\frac{1}{[I]} + \frac{1}{k_i}$ (18). So a replot of the reciprocal of these rate constants, $k_{app}$, versus reciprocal inactivator concentration, [I], is a straight line. The equilibrium constant for initial binding, $K_i$, and the

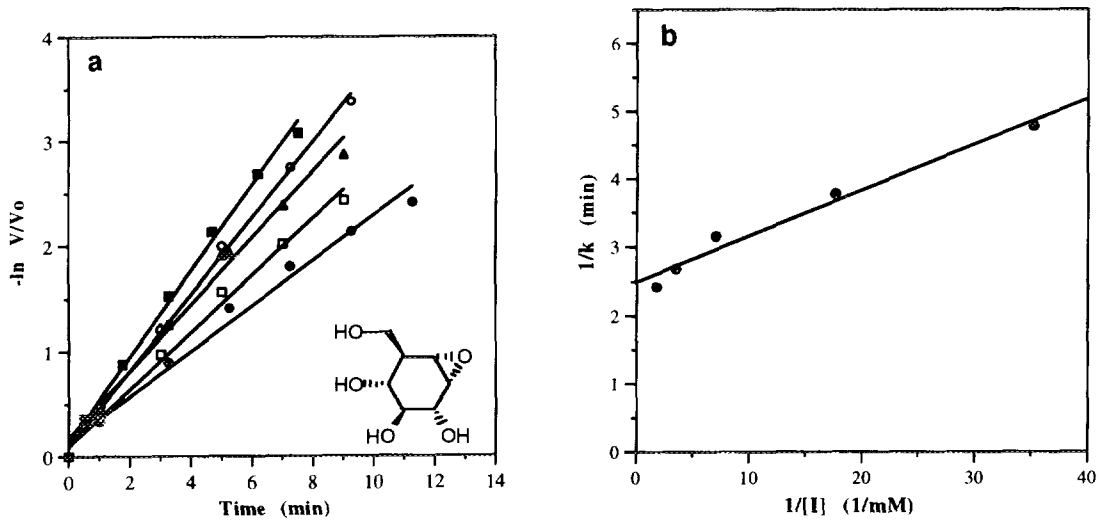


FIGURE 2. Inactivation of brewers yeast α-D-glucosidase by (1*R*,6*S*)-cyclophellitol. (a) Semi-logarithmic plot of residual activity versus time at the following concentrations: (■), 568 μM; (○), 284 μM; (▲), 142 μM; (□), 56.8 μM; (●), 28.4 μM. (b) Double-reciprocal plot of apparent first order rate constant from (a). Values are means of duplicate measurements.

TABLE 1. Comparisons of the kinetic parameters of cyclophellitols and conduritol epoxides

| inhibitor | enzyme | $K_i$ (mM) | $k_i$ ($min^{-1}$) | $k_i / K_i$ ($mM^{-1}\ min^{-1}$) | References |
|---|---|---|---|---|---|
| cyclophellitol | β-D-glucosidase, almonds | 0.34 | 2.38 | 7.00 | (10) |
| conduritol B epoxide | β-D-glucosidase, sweet almonds B | 1.7 | 0.13 | $7.6 \times 10^{-3}$ | (19) |
| (1*R*,6*S*)-cyclophellitol | α-D-glucosidase, brewers yeast | 0.0269 | 0.40 | 15.4 | this work |
| conduritol B epoxide | α-D-glucosidase, yeast, *S. cerevisieae* | 25 | 0.16 | $6.4 \times 10^{-3}$ | (1) |
| (1*R*,2*S*,6*S*)-cyclophellitol | α-D-mannosidase, jack beans | 0.120 | 2.85 | 23.8 | this work |
| conduritol F epoxide | α-D-mannosidase, jack beans | — | — | $8 \times 10^{-4}$ | (1) |

inactivation rate constant, $k_i$, can thus be obtained from the graph and the kinetic parameters are shown in Table 1. In order to investigate the irreversible binding between α-D-glucosidase and the (1*R*,6*S*)-diastereoisomer, we examined the influence of dialysis on the enzyme inhibition. As shown in Figure 3, inactivator-treated enzyme was also supressed after dialysis.

Similarly, the (1*R*,2*S*,6*S*)-diastereoisomer inactivates jack beans α-D-mannosidase according to pseudo-first order kinetics as shown in Figure 4 and the kinetic parameters obtained from the double reciprocal plot were shown in Table 1. As shown in Figure 5, the (1*R*,2*S*,6*S*)-diastereoisomer treated α-D-mannosidase was also suppressed after dialysis, showing irreversible binding of this compound with the enzyme.

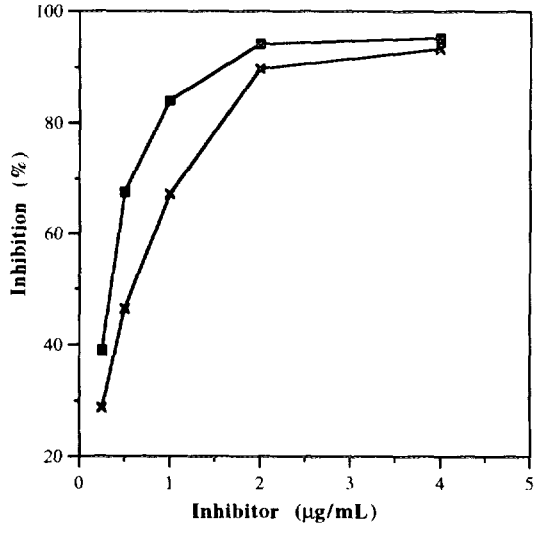


FIGURE 3. Effect of dialysis on brewers yeast α-D-glucosidase by (1*R*,6*S*)-cyclophellitol: (■) the control sample, (×) the dialysed sample.

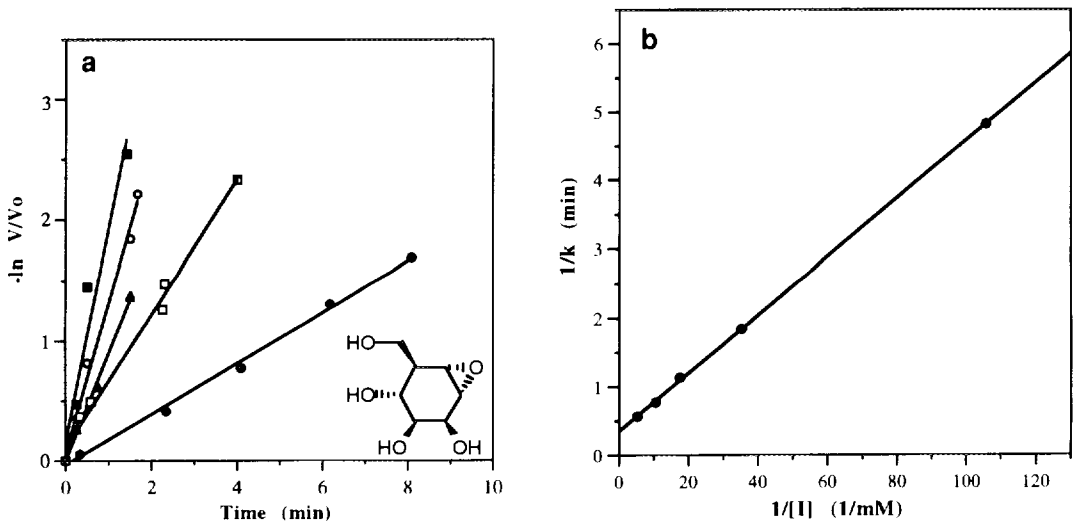


FIGURE 4. Inactivation of jack beans α-D-mannosidase by (1*R*,2*S*,6*S*)-cyclophellitol. (a) Semi-logarithmic plot of residual activity versus time at the following concentrations: (■), 189 μM; (○), 94.7 μM; (▲), 56.8 μM; (□), 28.4 μM; (●), 9.47 μM. (b) Double-reciprocal plot of apparent first order rate constant from (a). Values are means of duplicate measurements.

A comparison of the inactivation parameters obtained from cyclophellitol analogues and those from conduritol epoxides is shown in Table 1. Structurally, conduritol epoxides can be compared with cyclophellitol analogues, the difference lies only in the replacement of the C-5 hydroxymethyl group in cyclophellitol with a hydroxy group in conduritol epoxides. Concerning the dissociation constants of inactivation, $K_i$, the values for the cyclophellitols

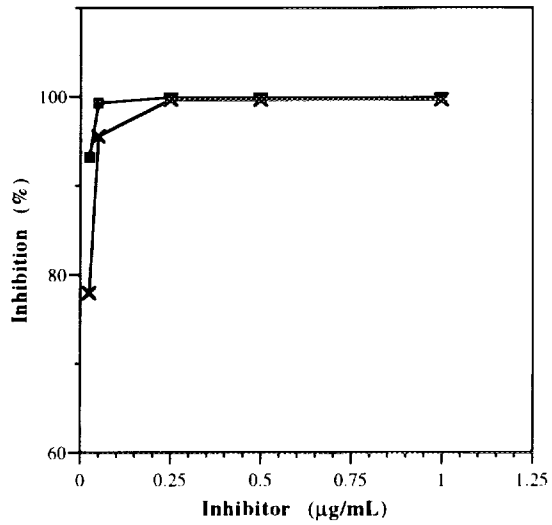


FIGURE 5. Effect of dialysis on jack beans α-D-mannosidase by (1*R*,2*S*,6*S*)-cyclophellitol: (■) the control sample, (×) the dialysed sample.

were much smaller than those of the corresponding conduritol epoxides, implying that the initial formation of the non-covalent enzyme-inactivator complex is a much favourable process in cyclophellitols. This difference can be attributed to the C-5 hydroxymethyl group which is a real analogue of hexose and therefore a better fitting of the active site.

It was reported that cyclophellitol is 92 fold more effective (based upon relative $k_i/K_i$ values) than conduritol B epoxide towards β-D-glucosidase from almonds (10). Our findings also showed that the (1*R*,6*S*)-diastereoisomer is *ca.* $2 \times 10^3$ fold more effective than conduritol B epoxide towards α-D-glucosidase from yeast, while the (1*R*,2*S*,6*S*)-diastereoisomer is *ca.* $3 \times 10^4$ fold more effective than conduritol F epoxide towards α-D-mannosidase from jack beans. These results revealed that cyclophellitol and its diastereoisomers are much potent inactivators than the conduritol epoxides.

In conclusion, further to our previous findings that cyclophellitols possess specific inhibitory activities towards glycosidases, we reported herein the irreversible properties and kinetic parameters of the (1*R*,6*S*)- and (1*R*,2*S*,6*S*)-diastereoisomers of cyclophellitols.

## ACKNOWLEDGMENTS

We thank the Croucher Foundation (to T. K. M. S.) and the Research Grant Council (to Y.-S. W.) for financial support.

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