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CYCLOPHELLITOL: A NATURALLY OCCURRING MECHANISM-BASED INACTIVATOR OF BETA-GLUCOSIDASES

S.G. Withers* and K. Umezawa+

Department of Chemistry, University of British Columbia,
Vancouver, B.C., Canada V6T 1Y6

+Department of Applied Chemistry, Faculty of Science and Technology,
Keio University, 3-14-1 Hiyoshi, Kohokuku, Yokahama 223, Japan

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The natural product cyclophellitol, isolated from the culture filtrate of a mushroom, *Phellinus sp.* is found to be a highly specific and effective irreversible inactivator of beta-glucosidases. It inactivates the beta-glucosidases from both almond emulsin and *Agrobacter sp.* according to pseudo-first order kinetics with inactivation constants of Ki = 0.34 mM, ki = 2.38 min^{-1}, and Ki = 0.055 mM, ki = 1.26 min^{-1} respectively. No reactivation of the inactivated enzyme is seen upon dialysis, thus providing evidence for the irreversibility of the inactivation. The high specificity of this inactivator is evidenced by the fact that even at very high (12 mM) concentrations of cyclophellitol, no inactivation of yeast alpha-glucosidase was observed, and only extremely slow ($t_{1/2}$ > 5 hours) inactivation of *E. coli* beta-galactosidase could be detected.

There has been considerable interest of late in the generation and testing of mechanism-based inhibitors of glycosidases. This interest is based not only upon the inherent usefulness of these compounds as probes of mechanism and active site structure of glycosidases (1,2), but also upon their utility in probing mechanisms of glycoprotein processing (3) and their therapeutic potential (4,5). Such compounds could be useful in the treatment of diabetes and obesity by interference with starch digestion (4) and may inhibit viral infections through interference with the normal processing of viral glycoprotein, thus decreasing infectivity (5).

Among the compounds recently described which act as mechanism-based inactivators are the glycosylmethyl triazenes (6), the 1',1'-difluoroalkyl glucosides and related compounds (7,8), the aziridines based upon galactose (9) and conduritol (10), and the 2-deoxy-2-fluoro-D-glycosides(11). However, the first class of mechanism based glycosidase inactivators to be described, over twenty years ago, were the conduritol epoxides such as conduritol B epoxide (**1**), an excellent covalent inactivator of both α- and β-glucosidases (12,13,14). This compound presumably binds at the active site in a similar mode to the glucoside substrate, the epoxide oxygen is protonated by the acid catalytic group, then the nucleophilic carboxylate attacks this activated

*To whom correspondence should be addressed.

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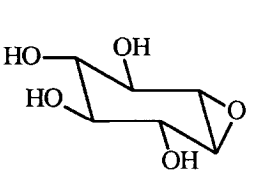
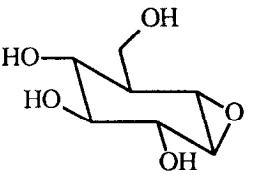


(1)



(2)

species forming a covalent derivative and inactivating the enzyme. Indeed such inactivators have been used to identify the putative active site nucleophiles in several glycosidases (13,14). However, there are several drawbacks to the use of this specific inhibitor, and the closely related aziridine analogue (10), all based upon the fact that such inhibitors lack the C-5 hydroxymethyl group present in the parent substrate, thus are truly analogues of xylose, not glucose. One consequence is that the epoxide has an element of symmetry which makes it an analogue of both an α-glucoside and a β-glucoside and indeed the conduritol B epoxide is a good inactivator of both α- and β-glucosidases. In addition, the relatively poor fit of these epoxides may allow some flexibility in their binding modes, thus the possibility that the amino acid side chain labelled may be other than that which ordinarily acts as the active site nucleophile. As was noted previously (10) both these problems would be solved through the synthesis of the corresponding epoxide inactivator (2) which also contains the C-5 hydroxymethyl group; however, the synthesis of such a molecule is a non-trivial matter. Surprisingly, this actual compound (named cyclophellitol) was recently isolated from culture filtrates of a mushroom, *Phellinus sp.*, and shown to act as an inhibitor of β-glucosidases (15,16). It presumably serves a defensive, antifeedant role. It has subsequently been chemically synthesised (17). In this paper we describe the characterisation of the inhibitory action of this material, showing that it is indeed a specific covalent inactivator of β-glucosidases which operates via the expected kinetic mechanism.

MATERIALS AND METHODS

Buffer chemicals and substrates were obtained from Sigma Chemical Company, as were the α-glucosidase (Type III from yeast), the almond β-glucosidase and the β-galactosidase (Grade IX from *Escherichia coli*). No additional purification was employed. The β-glucosidase is an enzyme originally isolated from *Agrobacter sp.* (previously typed as *Alcaligenes faecalis*) (18) and since cloned into *Escherichia coli* (19). This enzyme was isolated essentially as described previously (18). Cyclophellitol was isolated from a culture filtrate of *Phellinus sp.* as described previously (15).

Inactivation experiments were performed by incubating the enzyme in the appropriate buffer (see below) in the presence of cyclophellitol and any required metal ions. Aliquots (10 μL) were removed at appropriate time intervals and assayed for residual enzyme activity by dilution into a large volume (0.75 mL) of saturating concentrations of the corresponding nitrophenyl glycoside substrate in the same buffer system. This effectively halts 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 nitrophenolate release through the increase in absorbance at 400 nm. Pseudo-first order rate constants at each inactivator concentration were calculated by fitting the data to a first order equation, or by plotting the logarithm of the residual activity versus time. Replotting the reciprocal of these rate constants versus reciprocal inactivator concentration provided values for the dissociation constant K_i and the maximal inactivation rate constant k_i. Buffer systems, temperatures and substrates employed for the inactivation experiments for each enzyme were as follows: *Agrobacter* β-glucosidase, 50 mM

sodium phosphate buffer, pH 6.8, 37 ^{0}C, p-nitrophenyl β-D-glucopyranoside; sweet almond β-glucosidase, 20 mM sodium acetate, pH 6.8, 37 ^{0}C, p-nitrophenyl β-D-glucopyranoside; *Escherichia coli* β-galactosidase, 50 mM sodium phosphate, 1 mM Mg^{2+}, pH 7.0, 25 ^{0}C, p-nitrophenyl β-D-galactopyranoside; yeast α-glucosidase, 50 mM sodium phosphate, pH 6.8, 25 ^{0}C, p-nitrophenyl α-D-glucopyranoside.

RESULTS AND DISCUSSION

Cyclophellitol was shown to be an excellent time-dependent irreversible inactivator of both the *Agrobacter sp.* β-glucosidase and almond β-glucosidase. In both cases it showed saturable pseudo 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 *Agrobacter* β-glucosidase are presented in Figure 1 in the form of a logarithmic plot of residual activity versus time. All plots are cleanly linear, as required for first order kinetic behaviour, 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 K_i = 55 μM and an inactivation rate constant, k_i = 1.26 min^{-1}. Protection against inactivation was provided by the competitive inhibitor β-glucosyl

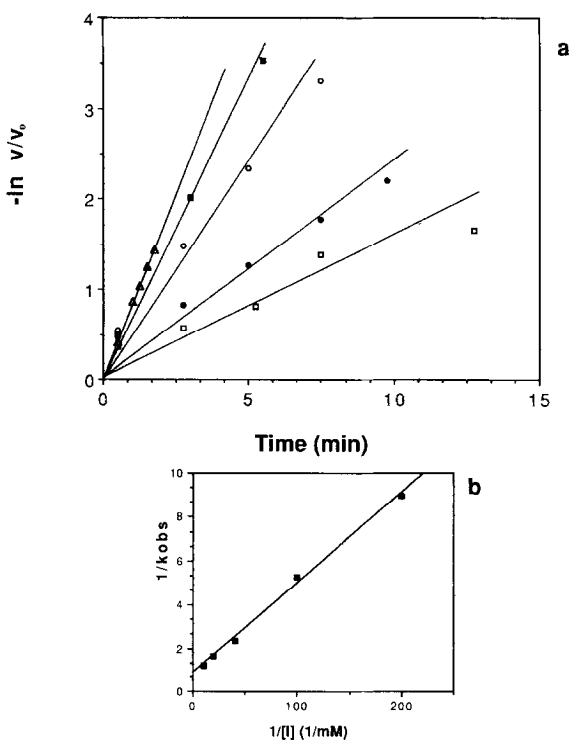


Figure 1. Inactivation of *Agrobacter* β-glucosidase by cyclophellitol.
a) Plot of ln residual activity ratio *versus* time; conditions as described in the text. Concentrations of cyclophellitol employed were: (Δ) 0.1 mM, (■) 0.05 mM, (○) 0.025 mM, (●) 0.01 mM, (□) 0.005 mM.
b) Replot of first order rate constants from a).

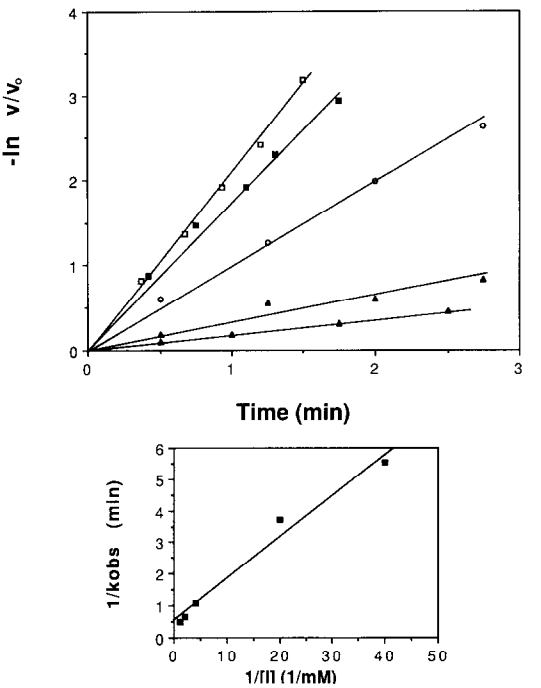


Figure 2. Inactivation of almond β-glucosidase by cyclophellitol.
a) Plot of ln residual activity ratio *versus* time; conditions as described in the text. Concentrations of cyclophellitol employed were: (❑) 0.9 mM, (■) 0.5 mM, (○) 0.25 mM, (▲) 0.05 mM, (Δ) 0.025 mM.
b) Replot of first order rate constants from a).

benzene, since addition of 11 mM glucosyl benzene (K_i = 3.4 mM) to an inactivation mixture containing 0.05 mM cyclophellitol reduced the rate of inactivation from 0.64 min^{-1} to 0.20 min^{-1}. It would be of considerable interest to compare the inactivation parameters obtained with those for the conduritol epoxide to assess the contribution of the hydroxymethyl group to the inactivation rate, but unfortunately such numbers have not been determined for this enzyme. However, the following data on the closely similar conduritol aziridine are available: K_i = 3.0 mM and k_i = 0.077 min^{-1} (10). Thus cyclophellitol is indeed considerably more effective (880 fold by comparison of k_i/K_i values) as an inactivator, pointing out the probable importance of these non-covalent interactions. Interestingly, a similar ratio (340 : 1) is found for the relative substrate specificities of this enzyme for p-nitrophenyl glucoside and p-nitrophenyl xyloside (S.G. Withers, unpublished results), two substrates which differ only in the presence of the C-5 hydroxymethyl group. This lends support to the proposal that cyclophellitol is indeed a mechanism-based inactivator which "recruits" the enzyme's catalytic apparatus in its mode of action.

Similar inactivation data were obtained for the almond β-glucosidase and these are shown in graphical form in Figure 2. Once again good pseudo-first order kinetic behaviour was observed, and inactivation constants of K_i = 0.34 mM and k_i = 2.38 min^{-1} were determined. β-Glucosyl benzene again provided protection against inactivation, an 11 mM concentration decreasing the rate of inactivation at 0.05 mM cyclophellitol from 0.30 min^{-1} to 0.19 min^{-1}. In this case it is possible to compare data with the parameters determined previously for conduritol B-epoxide with the

almond enzyme (20). However, since no attempt was made to separate the two isoenzymes in our study, results are compared with the data for the isoenzyme (B) which was the most rapidly inactivated by conduritol epoxide: K_i = 1.7 mM, k_i = 0.13 min^{-1}. Thus cyclophellitol is 92 fold more effective (based upon relative k_i/K_i values) than the conduritol epoxide. Again, this is consistent with the fact that p-nitrophenyl xyloside is a 50 fold poorer substrate for this enzyme than p-nitrophenyl glucoside (21).

In contrast to the above results, incubation of high (up to 12 mM) concentrations of cyclophellitol with yeast α-glucosidase resulted in no inactivation of the enzyme over 16 hours. This is particularly significant since both conduritol B-epoxide and conduritol aziridine were highly effective inactivators of yeast α-glucosidase (10,12), yielding inactivation rate constants of K_i = 25 mM, k_i = 0.16 min^{-1} and K_i = 9.5 mM, k_i = 0.39 min^{-1} respectively. Indeed the aziridine was a better inactivator of the α-glucosidase than the β-glucosidase. Clearly the presence of the equatorial hydroxymethyl substituent has introduced an absolute specificity regarding anomeric configuration. This is exactly as would be expected since the symmetry of the inhibitor has been broken. Incubation of a high (12.5 mM) concentration of cyclophellitol with *E. coli* β-galactosidase resulted in only 28% inactivation of the enzyme over a period of 130 minutes. This also is completely consistent with the specificity of β-galactosidase which is reported (22) to have only 1/10,000 the activity towards 2,4-dinitrophenyl glucoside as it has towards the corresponding galactoside.

The irreversibility of the inactivation was investigated by complete inactivation of *Agrobacter* β-glucosidase (2 mg mL^{-1}) with cyclophellitol (0.1 mM for 15 minutes), dialysis of this sample overnight (3 x 400 mL buffer, 0 ^{0}C), then incubation at 37 ^{0}C and assaying for return of activity. No enzyme activity could be recovered in this way, even when incubated over periods of up to 3 days in the presence of glucose (35 mM), glucosyl benzene (11 mM) or cellobiose (20 mM), ligands which have previously (23) been shown to be capable of reactivating certain inactivated glucosidases. Cyclophellitol also inhibited almond β-glucosidase irreversibly (16).

Cyclophellitol is therefore a highly specific and potent irreversible inactivator of β-glucosidases and should prove an extremely valuable tool for investigating the mechanisms of action of β-glucosidases, probing mechanisms of glycoprotein processing and possibly even as a therapeutic agent.

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