Carbohydrate Research, 250 (1993) 101–112
Elsevier Science Publishers B.V., Amsterdam

Suicide-substrate inactivation of β-galactosidase by diazomethyl β-D-galactopyranosyl ketone †

James N. BeMiller [a,b], Regina J. Gilson [b], Robert W. Myers [b] and Marcelo M. Santoro [b]

[a] *Whistler Center for Carbohydrate Research, 1160 Smith Hall, Purdue University, West Lafayette, Indiana 47907 (USA)*

[b] *Department of Chemistry and Biochemistry, Southern Illinois University at Carbondale, Illinois 62901 (USA)*

(Received November 10th, 1992; accepted in revised form June 14th, 1993)

ABSTRACT

Diazomethyl β-D-galactopyranosyl ketone (**1**) has been proven to be a mechanism-based, irreversible (suicide-substrate) inactivator of *Aspergillus oryzae* β-D-galactosidase, but not an inactivator of *E. coli lacZ* β-D-galactosidase. Compound **1** is stable in buffers of normal physiological pH. It is decomposed by H^+, but not by nucleophiles. Inactivation of *A. oryzae* β-D-galactosidase was proven to be mechanism-based and irreversible via experiments which showed that the enzyme could be protected from inactivation by a competitive inhibitor, neither diazomethyl β-D-glucopyranosyl ketone (**2**) nor diazomethyl α-D-galactopyranosyl ketone inactivated the enzyme and therefore inactivation is stereospecific, excess inhibitor could be separated from inactive enzyme without regain of activity and therefore it is bound irreversibly, and a second pulse of enzyme is inactivated at the same rate as enzyme inactivated to 95% activity by the first pulse. Diazomethyl β-D-glucopyranosyl ketone (**2**) inhibited sweet almond β-D-glucosidase.

INTRODUCTION

Many glycosidases are highly specific and have relatively low binding constants, allowing use of small amounts of substrate and, hence, allowing them to be used for complex carbohydrate structure analysis. It was the purpose of this research to evaluate the inhibition effectiveness of a compound, diazomethyl β-D-galactopyranosyl ketone, designed to be a suicide-substrate inactivator of β-galactosidases[1].

The design of inhibitors of any type for any enzyme requires some knowledge of the enzyme's active site as well as its mechanism of action (Fig. 1). Glycosidases catalyze hydrolysis of glycosidic bonds via general acid-base catalysis with either

* Corresponding author.

† Journal paper No. 12520 of the Purdue Agricultural Experiment Station.

SSDI 0008-6215(93)E0203-D

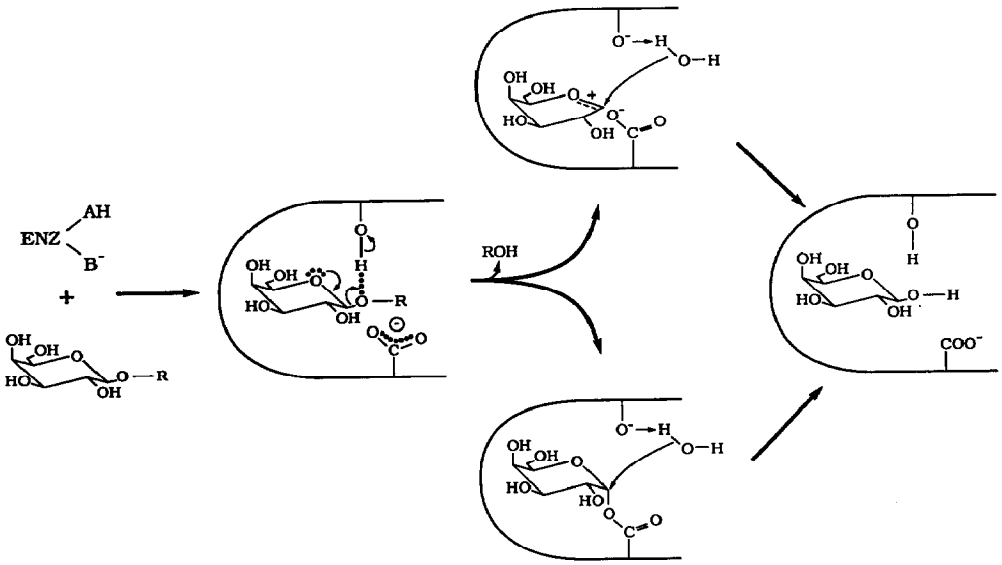

Fig. 1. Alternative proposed mechanisms of action of a retaining glycosidase, specifically *E. coli lacZ* β-D-galactosidase[2], the best understood glycosidase.

retention or inversion of configuration at C-1. Enzymes that retain configuration have been the most extensively studied and include *E. coli lacZ* β-D-galactosidase. The following features of the active site of the retaining glycosidases are important for catalysis: (1) their structures confine their substrates to rigid structural requirements; (2) their active sites are shielded from the aqueous surroundings so that molecules bind with their solvent cages removed; (3) they contain an acidic group (AH or AH^+) that is able to donate a proton to the glycosidic oxygen atom; subsequently, its conjugate base (A^- or AH) accepts a proton from an incoming nucleophile (usually water); and (4) they contain a nucleophile (B^-) that may contribute to cleavage of the glycosidic bond by electrostatically supporting the formation of the glycosyl carboxonium (oxocarbenium) ion transition state species and by stabilizing the same high-energy intermediate through reversible formation of a covalent glycosylenzyme species or a tight ion pair with the same steric constraints as those of a covalent intermediate. *E. coli lacZ* β-galactosidase requires Mg^{2+} ions[2–4] for activity.

Irreversible inhibitors of a specific target enzyme should find application in investigations of the mechanisms of glycosidases in in vivo biochemical studies, and as therapeutic agents. Several compounds inhibit glycosidases in the suicide-substrate manner[5–13]. However, general use these types of reagents is limited by their instability and/or their general reactivity with nucleophiles under physiological pH conditions[10].

Diazomethyl ketones are inert to irreversible nucleophilic attack in aqueous solution provided that low pH, high intensity light (400 nm), and certain transition

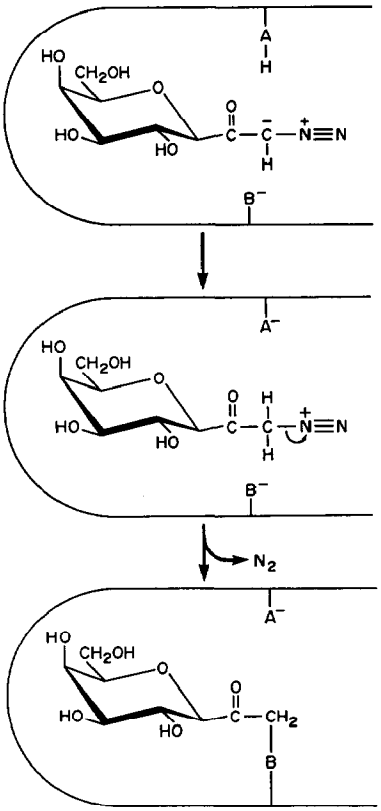

Fig. 2. Proposed mechanism of inactivation of *A. oryzae* β-D-galactosidase by diazomethyl β-D-galactopyranosyl ketone (**1**). The generated carbenium ion (real or transition state) could react with either A^- or B^-.

metals (e.g., Cu^{2+}) are avoided[15]. Their stability is a result of resonance stabilization of the diazo group via its conjugation with the carbonyl group. This property permits successful in vivo utilization of these inactivators[10].

This report describes the activity of a compound, diazomethyl β-D-galactopyranosyl ketone (**1**), designed to be a mechanism-based irreversible inhibitor of β-D-galactosidases[1]. Since glycosidases act via acid–base catalysis, it was hypothesized that **1** would be a suicide substrate of β-D-galactosidases reacting in a manner outlined in Fig. 2.

RESULTS AND DISCUSSION

Initial velocity vs. enzyme concentration.—A plot of initial velocity (v_i) vs. concentration of *A. oryzae* β-D-galactosidase in 0.1 M sodium phosphate buffer, pH 7.3, was linear over the range of concentrations used (3.837–38.37 μg/mL). A similar plot of v_i vs. concentration of the same enzyme in 0. 1 M sodium acetate

TABLE I

Acid stability of **1** [a]

pH	Buffer (100 mM)	$k_{app} \times 10^3$ (h^{-1})	$k_{1/2}$ (h)
7.3	Sodium phosphate	< 1.4	> 500
7.3	HEPES	< 1.4	> 500
4.5	Sodium acetate	3.6	194
3.5	*N*-Glycylglycine	26	26.6
3.0	*N*-Glycylglycine	106	6.54
2.5	Glycine	237	2.92
2.0	Glycine	808	0.86

[a] [**1**] = 50 μM.

buffer, pH 4.5, was linear over the concentration range 0.33–6.6 μg/mL. A graph of v_i vs. concentration of *E. coli lacZ* β-D-galactosidase in 0.1 M sodium phosphate buffer, pH 7.3, was linear over the range of concentrations used (1.98–19.8 μg/mL). A similar plot using sweet almond β-D-glucosidase in 0.1 M sodium acetate buffer, pH 4.5, was linear from 3.75 to 37.5 μg/mL.

Absorption spectra of diazomethyl β-D-galactopyranosyl ketone (**1**).—Absorption maxima of **1** occurred at 280 nm (peak), 350 nm (shoulder), and 251 nm (shoulder) in 0.1 M sodium acetate buffer, pH 4.5, and 0.1 M sodium phosphate buffer, pH 7.3. Extinction coefficients of **1** in 0.1 M sodium acetate buffer, pH 4.5, were 6609 M^{-1} cm^{-1} at 251 nm and 12207 M^{-1} cm^{-1} at 280 nm. The extinction coefficients in 0.1 M sodium phosphate buffer, pH 7.3, were 5892 M^{-1} cm^{-1} at 251 nm and 12535 M^{-1} cm^{-1} at 280 nm. The extinction coefficient at 350 nm in all solutions tested was 43 M^{-1} cm^{-1}. Extinction coefficients of **1** in water were reported previously by Myers and Lee[1].

Stability of **1**.—Ultraviolet radiation and certain transition metal ions effect decomposition of some diazoketones[15]. Thus, **1** was used in the absence of light and transition metal ions. Compound **1** (50.0 μM) was stable for at least 67 h in buffers of pH 7.3 and above; it, however, decomposed at pH 4.5 and below (Table I). The second order rate constant was 2.22×10^{-2} sec^{-1} M^{-1} for decomposition of **1** in the lower-pH buffers.

A good in vivo inhibitor must also be stable in the presence of nucleophiles. Compound **1** (75.0 μM) was stable in the presence of glutathione (10 mM; reduced form), NaBr (10 mM), and imidazole (10 mM) for at least 8 days in 0.1 M sodium phosphate buffer, pH 7.3, at 25°C in the absence of light.

Irreversible inactivation of β-galactosidases by **1**.—The proposed mechanism of β-D-galactosidase inactivation by **1** is given in Fig. 2. During initial studies, it was found that there was no loss of enzyme activity vide infra when **1** (20 mM) was incubated with *E. coli lacZ* β-D-galactosidase at its pH optimum, pH 7.3, for 24 h; the failure of **1** to inactivate this enzyme might have been due to the low acidity of

the active-site proton donor, believed to be a tyrosine residue[16,17] with an apparent pK_a of 8.5. It might also have been a consequence of *E. coli lacZ* β-D-glactosidase using Mg^{2+} rather than H^+ as an electrophile[2–4]. It was then surmised that the inhibitor might be effective against a glycosidase with a more acidic active-site proton donor. Therefore, the effectiveness of **1** as a mechanism-based inhibitor was tested against *A. oryzae* β-D-galactosidase with a pH optimum of 4.5. The ability to bind to the active site of an enzyme is an important criterion of an inactivator. Large concentrations of **1** were required before any inhibition could be detected; however, Lineweaver–Burk plots showed evidence of competitive reversible inhibition. A binding constant (K_i) of 38.8 ± 6.0 mM, which is within the same order of magnitude as the K_m of the enzyme for its natural substrate lactose (18 mM)[18], demonstrated that the enzyme recognizes a (β-D-galactopyranosyl)formyl group equally as well as an *O*-(β-D-galactopyranosyl) group.

The essentials for suicide inactivation were examined by incubating *A. oryzae* β-D-galactosidase with **1**, then assaying for remaining enzyme activity. First, the enzyme was found to be completely stable (100% activity) for at least 3 days at 25°C in the absence of inhibitor. Then, the enzyme was incubated with **1** in the same buffer. Aliquots transferred from the incubation medium to the reaction medium were diluted 100×, thereby negating the effects of any reversible inhibition occurring during the enzyme assay. First-order inactivation of the enzyme over at least three half-lives was determined by plotting ln (percent remaining enzyme activity) against time. There was no evidence of a lag phase, indicating no time-dependent diffusion of activated inhibitor from the active site to react elsewhere. The K_i value was calculated to be 30.2 ± 4.0 mM, which agrees well with the value determined by treating **1** as a reversible inhibitor. The k_{max} (the maximum rate of inactivation) for the reaction was 0.556 ± 0.016 min^{-1} and the $t_{1/2}$ was 74.7 ± 2.2 s, indicating a relatively fast reaction.

Another criterion for active site-directed, irreversible inhibition is that the enzyme must be protected from inactivation by a substrate or a competitive inhibitor. No competitive inhibitors of *A. oryzae* β-D-galactosidase have been reported. However, Sinnott and Smith[19] describe protection of *E. coli lacZ* β-D-galactosidase by methyl 1-thio-β-D-galactopyranoside, which acts as a reversible inhibitor. Phenyl 1-thio-β-D-galactopyranoside (PTG) (K_i = 2.82 ± 0.16 mM with *A. oryzae* β-D-galactosidase) was used in this work because it was presumed that it would bind well because of its hydrophobic aglycon[20]. The rate of inactivation decreased as the concentration of PTG increased while the concentration of **1** remained constant; again, inactivation displayed first-order kinetics without a lag phase. When the concentration of the PTG was held constant and the concentration of **1** varied, a K_i of 2.24 ± 0.16 mM for PTG was determined, a value consistent with the K_i calculated from reversible inhibition data.

The results of the two experiments, each varying the concentration of either **1** or PTG while the concentration of the other was held constant, indicate that the enzyme could be protected from irreversible inhibition by a competitive inhibitor

and that, therefore, the reaction with **1** took place at the active site, i.e., that **1** is an active site-directed inhibitor of *A. oryzae* β-D-galactosidase.

Additional experiments were performed to establish that the enzyme is suicidal in the presence of **1**. EDTA (5 mM) was added to an incubation mixture of **1** (6 mM) and *A. oryzae* β-D-galactosidase to chelate any divalent metal ions present in order to demonstrate that inactivation is not due to general labeling as a result of reaction of **1** with a metal ion to form a reactive metal-complexed carbene. No change in k_{app} was found, indicating that nonspecific labeling was not involved.

A. oryzae β-D-galactosidase was incubated with diazomethyl β-D-glucopyranosyl ketone (87 mM) in place of its galactose counterpart; no inactivation of the enzyme was observed over 50 h. This result establishes the likelihood that the enzyme is stereospecific for β-D-galactopyranosyl compounds, again inferring that **1** binds to the active site.

Isolation and reactivation of inactivated Aspergillus oryzae *β-D-galactosidase.*—*A. oryzae* β-D-galactosidase was incubated with an excess of **1** at pH 4.5. Excess **1** was separated from completely inactivated enzyme by size-exclusion chromatography; fractions constituting the enzyme inactivated by **1** (peak at the void volume) were pooled, and reactivation of the enzyme was followed. Using the combined absorbances of the fractions and the concentrations of the compounds before gel filtration, a ratio of moles of compound recovered to moles applied to the column was determined. Recovery of the protein was found to be 96.5% and that of unreacted **1**, 97. 1%. This result demonstrates that unreacted **1** could be separated from the enzyme without the enzyme regaining any of its activity, i.e., that reacted inhibitor was irreversibly bound to the enzyme. Recovery of activity was followed in acetate buffer, pH 4.5. After 75 h, 17% of the initial enzyme activity was recovered. The k_b (rate constant of reactivation) was found to be 2.50×10^{-3} h^{-1} and the $t_{1/2}$ 277 h. Recovery is believed to be due to slow hydrolysis of enzyme–inhibitor covalent compound which is presumed to be an ester[21]. *E. coli lacZ* β-D-galactosidase acting on a β-galactoside substrate forms a galactosyl ester of Glu-461[22].

Mechanism and specificity of the irreversible inhibition.—To investigate the theoretical possibility that *A. oryzae* β-D-galactosidase converts **1** into a reactive substance (a "hot" carbocation/carboxonium ion) that is released from the active site, diffuses away, and inactivates the enzyme by reacting with one of its nucleophilic groups at another site[23], two experiments were done. (*a*) On the assumption that a nucleophile would trap an electrophile resulting in a decrease in k_{app}, nucleophiles were added to the incubation solution. No change in k_{app} was observed. (*b*) After an incubation period in which 95% of the enzyme was inactivated by an excess of **1**, a second pulse of enzyme was added to the inhibition medium. If the theoretically possible mechanism were operative, or if there had been a spontaneous production of active inhibitor with time, an initial lag period [revealed in a ln (percent initial enzyme activity) vs. time plot] and an increased rate of inactivation should have been found. Neither were.

Incubations of **1** (25 mM) with *A. oryzae* β-D-galactosidase in 0.1 M sodium phosphate buffer, pH 7.3, gave a half-life of the enzyme of 1.15 h (k_{app} 0.603 h^{-1}). Incubations of **1** (18 mM) with sweet almond β-D-glucosidase in 0.1 M sodium acetate buffer, pH 4.5, resulted in a 68% decrease in enzyme activity over 6 days; the enzyme alone in the same buffer at 25°C lost no activity over the same time period. Inactivation of sweet almond β-D-glucosidase by **1** is probably due to the enzyme's non-specificity[24].

Incubations of **2** (25 and 100 mM, 10 times the concentrations of **1** used with *A. oryzae* β-D-galactosidase) with sweet almond β-D-glucosidase in 0.1 M sodium acetate buffer, pH 4.5, gave a half-life of the enzyme of 1.59 h and a k_{max} of 0.44 h^{-1}. The K_i determined for **2** was 172 mM. This work demonstrates the applicability of diazomethyl glycopyranosyl ketones as specific suicide-substrate inactivators of other glycosidases.

The k_{obs} for proton-mediated, non-enzymic decomposition of **1** at pH 4.5 was 7.02×10^{-7} s^{-1}, whereas the k_{max} for enzymic inactivation at the same pH was 9.27×10^{-3} s^{-1}; thus, the rate enhancement for the latter reaction is at least 10^4 under these conditions. This is in the range of rate enhancement for enzyme-catalyzed hydrolysis of glycosidases due to acid-catalyzed assistance of aglycon departure[25].

EXPERIMENTAL

Substrates.—*o*-Nitrophenyl β-D-galactopyranoside (ONP-βGal), *o*-nitrophenyl β-D-glucopyranoside (ONP-βGlc), and *p*-nitrophenyl β-D-glucopyranoside (PNP-βGlc) were purchased from Sigma Chemical Co., St. Louis, Missouri. All reagents were of the highest grade available and were used as purchased.

Instrumentation and inhibitor and buffer solutions.—Spectrophotometric measurements were made on a Beckman Acta MVI recording spectrophotometer. Water maintained at 25.0 ± 0.1°C was passed through the cuvette holder by means of a refrigerated bath circulator. Diazomethyl β-D-galactopyranosyl ketone (**1**) was prepared as reported[1]. All buffer solutions were prepared in deionized-distilled water; all pH values were determined at 25°C.

Extinction coefficient of o-nitrophenol.—The extinction coefficient of *o*-nitrophenol (ONP) was determined by serially diluting a stock solution (0.72 mM) in both 0.1 M sodium phosphate buffer, pH 7.3, and 0.1 M sodium acetate buffer, pH 4.5. The extinction coefficients at 410 nm and 25°C of ONP were determined to be 3973 M^{-1} cm^{-1} in sodium phosphate buffer, pH 7.3, and 574 M^{-1} cm^{-1} in sodium acetate buffer, pH 4.5.

Enzymes.—β-D-Galactosidase (β-D-galactoside-galactohydrolase; EC 3.2.1.23) from *A. oryzae* was obtained as a suspension (4.95 mg/mL) in 3.5 M ammonium sulfate and 20 mM phosphate, pH 6.5, from Boehringer Mannheim, Indianapolis, Indiana. An aliquot of enzyme was added to a substrate solution at pH 7.3 or 4.5 to begin hydrolysis of ONP-βGal, which was followed spectrophotometrically by

measuring the change in A_{410} at 25°C. (a) At pH 7.3: 50.0 μL of the enzyme suspension was added to 3.00 mL of 0.1 M sodium phosphate buffer, pH 7.3. The absorbance of this stock solution was determined at 280 nm and 25°C. Five concentrations of the enzyme were achieved by adding appropriate volumes of the enzyme stock solution and buffer [final assay volume 2.50 mL; final concentration = 0.80 mM ($\sim 1 \times K_m$) ONP-βGal and 1.0 mM $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.3]. (b) At pH 4.5: 10 μL of the enzyme suspension was added to 0.75 mL of 0.1 M sodium acetate buffer, pH 4.5. The absorbance of this enzyme stock solution was determined at 280 nm and 25°C. Six concentrations of the enzyme were achieved by adding appropriate volumes of the enzyme stock solution and buffer [final assay volume 2.50 mL; final concentration = 10.0 mM ($\sim 5 \times K_m$) ONP-βGal in 0.1 M sodium acetate buffer, pH 4.5].

β-D-Galactosidase from *E. coli lacZ* (Grade X, molecular weight: 540 000, extinction coefficient: $\epsilon(1\%) = 20.9$ at 280 nm) was obtained as a suspension (37 mg/mL) in 1.7 M ammonium sulfate, 0.01 M Tris · HCl, and 1.0 mM $MgCl_2$, pH 7.3, from Sigma Chemical Co., St. Louis, Missouri. The enzyme suspension (25.0 μL) was added to 5.00 mL of 0.1 M sodium phosphate buffer, pH 7.3. The concentration of this stock solution was determined spectrophotometrically at 280 nm and 25°C. Five concentrations of the enzyme were made by adding appropriate volumes of the enzyme stock solution and buffer [final assay volume 2.50 mL; final concentration = 0.80 mM ($\sim 10 \times K_m$) ONP-βGal and 1.0 mM $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.3]. An aliquot of the enzyme stock solution was added to the assay solution to begin the reaction which was followed spectrophotometrically by measuring the change in A_{410} at 25°C.

β-D-Glucosidase (β-D-glucoside-glucohydrolase; EC 3.2.1.21) from sweet almonds (Type I) was obtained from Sigma Chemical Co., St. Louis, Missouri. The enzyme (0.40 mg) was added to 1.00 mL of 0.1 M sodium acetate buffer, pH 4.5. The absorbance of this enzyme stock solution was determined spectrophotometrically at 280 nm and 25°C. Five concentrations of enzyme were achieved by adding appropriate volumes of the enzyme stock solution to a solution of PNP-βGlc [final assay volume = 1.0 mL; final concentration = 30.0 mM ($\sim 6 \times K_m$) in 0.1 M sodium acetate buffer, pH 4.5]. The production of *p*-nitrophenol (PNP) from PNP-βGlc was followed spectrophotometrically by measuring the change in A_{410} at 25°C.

Initial velocity vs. enzyme concentration (v_i vs. [E]).—Initial velocities were plotted as a function of enzyme concentration, with 0.0 min^{-1} (velocity) at [E] = 0.00 M represented as a point. All concentrations of all enzymes used subsequently gave velocities that were within the linear range.

Absorption spectrum of **1**.—Three solutions of **1**, each 18.01 mM, were made in 0.1 M sodium phosphate buffer, pH 7.3, 0.1 M sodium acetate buffer, pH 4.5, and deionized-distilled water. Each solution was scanned from 500 to 200 nm at a temperature of 30°C. These three solutions were diluted to 90.05 μM and again scanned from 500 to 200 nm at 30°C.

Extinction coefficients of **1**.—Absorbances at 280 and 251 nm of **1** (89.85 μM) in both 0.1 M sodium phosphate buffer, pH 7.3, and 0.1 M sodium acetate buffer, pH 4.5, were determined at 25°C. Duplicate solutions were prepared in almost total darkness and absorbances were determined without prior exposure to light. Extinction coefficients were determined using the Beer–Lambert law.

Determination of Beer–Lambert region of **1**.—Duplicate stock solutions of **1** (90 μM) in both 0.1 M sodium phosphate buffer, pH 7.3, and 0.1 M sodium acetate buffer, pH 4.5, were serially diluted and the absorbances were determined spectrophotometrically at 280 and 251 nm. The linear range of concentration vs. absorbance plots were determined by linear least-squares treatment of the data. Both A_{280} vs. concentration and A_{251} vs. concentration plots were linear over the concentration range used (15–90 μM).

Stability of **1**.—The stability of **1** was determined as a function of pH by following changes with time in A_{280} of 50 μM solutions at 25°C in the following buffers: 0.1 M glycine (pH 2.0, 2.5, and 9.0), 0.1 M *N*-glycylglycine (pH 3.0 and 3.5), 0.1 M sodium acetate (pH 4.5), 0.1 M sodium phosphate (pH 7.3), and 0.1 M HEPES (pH 7.3). Apparent first-order rate constants for the decomposition of **1** were obtained by linear least-squares treatment of ln (percent initial absorbance at 280 nm) vs. time plots.

The stability of **1** in the presence of nucleophiles was determined by following changes with time at A_{280} of 75 μM solutions (25°C) in 0.1 M sodium phosphate buffer, pH 7.3, containing either NaBr (10 mM), glutathione (10 mM; reduced form), or imidazole (10 mM) and in 0.1 M sodium acetate buffer, pH 4.5, containing either NaBr (10 mM) or glutathione (10 mM; reduced form).

Reversible inhibition activity assays.—A. oryzae β-D-Galactosidase: Production of ONP from ONP-βGal (K_m 1.79 ± 0.11 mM; [S] = ~ 0.5, 1, 2, 4 × K_m) in the presence of various concentrations (0, 12, 24, 36, and 48 mM) of **1** in 0.1 M sodium acetate buffer, pH 4.5, was determined spectrophotometrically at 410 nm and 25°C. The reaction was initiated by addition of enzyme (~ 1.3 μg); total reaction volume 1.0 mL.

E. coli lacZ β-D-galactosidase: production of ONP from ONP-βGal (K_m 86.5 ± 2.8 μM; [S] = ~ 0.5, 1, 2, 4 × K_m) in the presence of various concentrations of **1** and 1.0 mM $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.3, was determined spectrophotometrically at 410 nm and 25°C. The reaction was initiated by addition of enzyme (~ 1.2 μg); total reaction volume 1.0 mL.

Sweet almond β-D-glucosidase: production of PNP from PNP-βGlc or ONP from ONP-βGlc (for both: K_m 4.73 ± 0.29 mM; [S] = ~ 0.5, 1, 2, 4 × K_m) in the presence of various concentrations of **1** and 0.1 M sodium acetate buffer, pH 4.5 or 5.0, was determined spectrophotometrically at 410 nm and 25°C. The reaction was initiated by addition of enzyme (~ 70 μg); total reaction volume 2.5 mL.

Kinetics of enzyme inactivation.—A. oryzae β-D-galactosidase: solutions (total volume 150 μL in microfuge tubes) of enzyme (12.4 μg) and varying concentrations (0, 3, 6, 9, 12, and 15 mM) of **1** in 0.1 M sodium acetate buffer, pH 4.5, were

incubated in the dark at 25°C. Reaction was initiated by addition of **1** to the enzyme stock solution. Measurements of 100% activity were made within 30 s of admixture by removing aliquots (10 μL) and adding them to an assay mixture of ONP-βGal (10.0 mM; $\sim 5 \times K_m$) [total assay volume = 1.0 mL; 100× dilution]. Additional aliquots (10 μL) were removed at various times thereafter and assayed as described. The K_i value was determined from a plot of k_{app}^{-1} (1/slope of ln activity vs. time for each concentration of **1**) vs. [**1**].

Inactivation constants were also determined in the presence of phenyl 1-thio-β-D-galactopyranoside (PTG; $K_i = 2.82 \pm 0.163$ μM; 0, 3, 6, 12, and 18 mM when used as a competitive inhibitor), EDTA (5 mM), and NaBr (10 mM). Diazomethyl β-D-glucopyranosyl ketone (**2**) (87 mM) was also used in place of **1**.

Sodium phosphate buffer (0.1 M; pH 7.3) was also used as a medium for inactivations. Solutions (total volume = 0.30 mL in cuvettes) of enzymes (50 μg) and **1** (25 mM) in buffer were incubated in the dark at 25°C. The reaction was initiated by addition of **1** to the enzyme stock solution. 100% Activity was determined as before; and at various times, aliquots (50 μL) were removed and diluted (20×) into a standard assay mixture of 10 mM ($\sim 10 \times K_m$) ONP-βGal (final assay volume = 1.0 mL).

E. coli lacZ β-D-galactosidase: a solution of enzyme (1.5 mg), **1** (20 mM), and $MgCl_2$ (1.0 mM) was incubated in the dark at 25°C in 0.1 M sodium phosphate buffer, pH 7.3 (total volume = 1.0 mL). The reaction was initiated by addition of **1** to the enzyme stock solution. 100% Activity was determined as before; and at various times, aliquots (25 μL) were removed and diluted 100× into a standard assay mixture of 0.8 mM ($\sim 10 \times K_m$) ONP-βGal (final assay volume = 2.5 mL).

Sweet almond β-D-glucosidase: solutions of enzyme (0.6 mg) and **2** (25 and 100 mM) were incubated in the dark at 25°C in 0.1 M sodium acetate buffer, pH 4.5 (total volume = 150 μL in microfuge tubes). The reaction was initiated by addition of **2** to the enzyme stock solution. 100% Activity was determined as before, and at various times aliquots (10 μL) were removed and diluted 100× into a standard assay mixture of 30.0 mM ($\sim 6 \times K_m$) ONP-βGlc (final assay volume = 1.0 mL).

A solution of enzyme (0.5 mg) and **1** (18 mM) was incubated in the dark at 25°C in 0.1 M sodium acetate buffer, pH 4.5 (total volume = 150 μL in a microfuge tube). The reaction was initiated by addition of **1**. 100% Activity was determined as before and at various times aliquots (10 μL) were removed and diluted 100× into a standard assay mixture of 30.0 mM ($\sim 6 \times K_m$) PNP-βGlc (final assay volume = 1.0 mL).

Reactivation of inactivated β-D-galactosidase (size-exclusion chromatography).— *A. oryzae* β-D-galactosidase (200 μg) in 0.1 M sodium phosphate buffer, pH 4.5, was inactivated with **1** to 0.5% residual activity (40 min). An aliquot (100 μL) of this solution containing 165 μg of enzyme was introduced onto a Sephadex G-25 column (100–300 mesh; 0.6 × 58.5 cm; V_t, 16.54 mL; V_0, 6.5 mL; flow rate, 0.33 mL/min; operating pressure, 27.0 cm; temperature, 25°C; buffer, 0.1 M sodium acetate). Absorbances (280 nm) of the fractions (0.5 mL) were determined. Three

fractions appearing in the void volume contained inactivated enzyme and were devoid of free **1**; they were pooled (total volume = 1.33 mL). Aliquots (25 μL) were assayed under standard conditions for recovery of enzyme activity with time by determining the production of ONP (A_{410}) from ONP-βGal (10 mM) in 0.1 M sodium acetate buffer, pH 4.5, at 25°C.

Addition of a second pulse of enzyme inactivation medium containing 1 (determination of mechanism-based irreversible inhibition).—A suspension (3 μL; ~ 15 μg) of *A. oryzae* β-D-galactosidase was added to a 6 mM solution of **1** in 0.1 M sodium acetate buffer, pH 4.5. Aliquots (10 μL) were taken at various times and assayed for enzyme activity by determining the production of ONP (A_{410}) from 10 mM ONP-βGal in 0.1 M sodium acetate buffer, pH 4.5, at 25°C. At 32 min, the enzyme was inactivated to 5.2% of its original activity. At 38 min (~ 4.2 half-lives) after addition of the initial pulse, a second pulse (3 μL; ~ 15 μg) of enzyme suspension was added from the same stock and aliquots were assayed for enzyme activity by the same method.

Inactivation of 1 as a function of pH.—Compound **1** (6 mM) and *A. oryzae* β-D-galactosidase (25 μg) solutions were incubated at 25°C in total darkness in 0.1 M sodium phosphate buffers, pH 6.0 and 8.0, and 0.1 M *N*-glycylglycine buffer, pH 3.0 (total volume = 150 μL in microfuge tubes). Reaction was initiated by addition of stock solutions of **1**, each made separately in each of the three buffers. 100% Activity was determined as before; and at time intervals, aliquots (10 μL) were removed and diluted 100 × with a standard assay medium, viz., 10 mM (~ $5 \times K_m$) ONP-βGal in 0.1 M sodium acetate buffer, pH 4.5.

ACKNOWLEDGMENTS

The authors acknowledge the help of Professor D.W. Bolen and the financial support of NIH and the Office of Research and Development, Southern Illinois University at Carbondale.

REFERENCES

1 R.W. Myers and Y.C. Lee, *Carbohydr. Res.*, 152 (1986) 143–158.
2 M.L. Sinnott, S.G. Withers, and O.M. Viratelle, *Biochem. J.*, 175 (1978) 539–546.
3 G. Legler and M. Herrchen, *Carbohydr. Res.*, 116 (1983) 95–103.
4 T. Selwood and M.L. Sinnott, *Biochem. J.*, 268 (1990) 317–323.
5 M. Brockhaus and J. Lehmann, *Carbohydr. Res.*, 63 (1978) 301–306.
6 M. Brockhaus and J. Lehmann, *FEBS Lett.*, 62 (1976) 154–156.
7 M. Brockhaus, H. Fritz, and J. Lehmann, *Carbohydr. Res.*, 68 (1979) 225–230.
8 P.J. Marshall, M.L. Sinnott, P.J. Smith, and D. Widdows, *J. Chem. Soc., Perkins Trans. 1*, (1981) 366–376.
9 G. Legler and M. Herrchen, *FEBS Lett.*, 135 (1981) 139–144.
10 G. Legler, *Methods Enzymol.*, 46 (1977) 368–381.
11 M.K. Tong and B. Ganem, *J. Am. Chem. Soc.*, 110 (1987) 312–313.
12 S. Halazy, C. Danzin, A. Ehrard, and F. Gerhart, *J. Am. Chem. Soc.*, 111 (1989) 3484–3485.
13 S.G. Withers and I.P. Street, *ACS Symp. Ser.*, 399 (1989) 597–607.

14 S.G. Withers, R.A. Warren, Jr., I.P. Street, K. Rupitz, J.B. Kempton, and R. Aebersold, *J. Am. Chem. Soc.*, 112 (1990) 5887–5889.
15 M. Regitz and G. Mass, *Diazo Compounds, Properties and Synthesis*, Academic Press, Orlando, FL, 1986, pp 23–29; 97; 189–191.
16 A.V. Fowler and P.J. Smith, *J. Biol. Chem.*, 258 (1983) 10204–10207.
17 J.-P. Tenu, O.M. Viratelle, J. Garnier, and J.M. Yon, *Eur. J. Biochem.*, 20 (1971) 363–370.
18 Y. Tanaka, A. Kagamiishi, A. Kiuchi, and T. Horiuchi, *J. Biochem.*, 77 (1975) 241–247.
19 M.L. Sinnott and P.J. Smith, *Biochem. J.*, 175 (1978) 525–538.
20 C.K. DeBruyne and M. Yde, *Carbohydr. Res.*, 56 (1977) 153–164.
21 M.L. Sinnott and I.J.L. Souchard, *Biochem. J.*, 133 (193) 89–98.
22 M. Herrchen and G. Legler, *Eur. J. Biochem.*, 138 (1984) 527–531.
23 R.H. Abeles and A.L. Maycock, *Acc. Chem. Res.*, 9 (1976) 313–319.
24 S. Veibel, in J.B. Sumner and K. Myrback (Eds.), *The Enzymes*, Vol. 1, Academic Press, New York, 1950, pp 593–599.
25 C.R. Clark and R.W. Hay, *J. Chem. Soc., Perkins Trans. 2*, (1973) 1943–1947.