Bromoketone *C*-Glycosides, a New Class of β-Glucanase Inactivators†

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Received May 6, 1998

Abstract: Although reliable methods have been developed for the labeling of the active site nucleophiles in glycosidases, no such reliable method has been developed for the identification of the acid/base catalyst. To address this problem two novel bromoketone affinity labels based on a β-*C*-glucoside (**6**), and a β-*C*-cellobioside (**9**), have been synthesized via a chemoenzymatic process and tested as inactivators of the β-glucosidase from *Agrobacterium* sp. and the β-glucanases from *Cellulomonas fimi*. The β-glucosidase was inactivated by **6** in a time-dependent manner according to kinetic parameters of $k_i = 0.01$ min^{-1} and $K_I = 3.1$ mM. Electrospray ionization mass spectrometric analysis revealed that multiple labeling of the enzyme had occurred. The β-endoglucanases, CenA and CenD, were inactivated stoicheometrically by **9** according to kinetic parameters of $k_i = 0.0155$ min^{-1}; $K_I = 0.35$ mM and $k_i = 0.01$ min^{-1}; $K_I = 6.0$ mM, respectively. These should therefore prove to be valuable reagents for the labeling of glycosidases.

Introduction

There has been widespread interest in glycosidases in recent years, largely due to their role in a multitude of biological and industrial processes. In particular, enzymes such as cellulases and amylases are important for the industrial degradation of biomass. Microorganisms that degrade cellulose generally secrete many different cellulases which act synergistically to hydrolyze the substrate. One organism, *Cellulomonas fimi*, produces an array of cellulases when grown on cellulose. Four β-endoglucanases (CenA, CenB, CenC, and CenD),[1−4] two exoglucanases (CbhA and CbhB),[5,6] and an exoglucanase/xylanase (Cex)[7] have been cloned and expressed in *Escherichia coli*.

Glycosidases can be classified as "retaining" or "inverting" depending upon the anomeric configuration of the initially formed product with respect to the substrate. Both types possess two catalytic carboxylic acid residues within the active site. Retaining glycosidases employ a "double-displacement mechanism" in which, in the first step, one residue provides general acid catalysis and the other provides nucleophilic catalysis to form a covalent glycosyl−enzyme intermediate.[8,9] In the second step, general base-catalyzed hydrolysis of this intermediate yields the product with retention of anomeric configuration. Inverting glycosidases use a single-step mechanism in which general base catalysis facilitates direct attack by water at the anomeric center, with general acid-catalyzed departure of the aglycon. All steps proceed via transition states with substantial oxocarbenium ion character.

Structural information, especially with respect to the identities of active site residues, is a key requirement for the understanding of these important enzymes and also provides the basis for engineering of new proteins with modified or improved function. A variety of mechanism-based inhibitors and affinity labels has been developed to label and identify catalytically important residues in this class of enzymes.[10,11] The catalytic nucleophiles in many cellulases, including Cex, have been labeled using 2-deoxy-2-fluoro-β-D-glycosides and their identities subsequently determined by peptide mapping.[12−16] In other cases the catalytic nucleophile has been labeled and identified using epoxyalkyl-β-D-xylosides and cellobiosides.[17,18]

Affinity labels have also been used successfully in the labeling and identification of the acid/base catalyst in some cellulases and xylanases. Notable successes include the identification of the acid/base catalytic residue in Cex with *N*-bromoacetyl-β-D-cellobiosylamine[19] and the determination of the crystal

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† Abbreviations: *C*-glycoside, carbon glycoside; β-PNPG, 4-nitrophenyl-β-D-glucopyranoside; β-DNPC, 2,4-dinitrophenyl-β-D-cellobioside; β-PNPC, 4-nitrophenyl-β-D-cellobioside; TLC, thin-layer chromatography; LC/MS, liquid chromatographic/mass spectrometric; ESMS, electrospray mass spectrometry; IPTG, isopropylthio-β-D-glucopyranoside.

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S0002-7863(98)01580-7 CCC: $15.00
Published on Web 09/23/1998

structure of the endo-β(1,4)-xylanase II from *Trichoderma reesei* in which the putative acid/base catalyst was covalently labeled with an epoxyalkyl-β-D-xyloside.[17] *N*-Bromoacetylglycosylamines have the advantage that they are inert toward enzyme-catalyzed hydrolysis, a potential problem in many other O-linked affinity labels, and they have been used with varying success. Keresztessy et al. have reported the successful labeling and identification of the putative acid/base catalyst in the β-glucosidase from *Manihot esculenta Crantz* using *N*-bromoacetyl-β-D-glucosylamine.[20] However, this compound was found to cause unselective, multiple labeling in the β-glucosidase from *Agrobacterium* sp.[21] Furthermore, in the case of *E. coli* β-galactosidase, *N*-bromoacetyl-β-D-galactosylamine was found to label a single active site residue, Met-502, which was, however, later shown not to participate in catalysis.[22]

Clearly, there is a need for new affinity labels with improved selectivity. Here we report the synthesis of novel cellobioside and glucoside affinity labels, using chemoenzymatic methods, which are designed to label enzyme acid/base catalysts. These new affinity labels **6** and **9** (Scheme 1) incorporate an α-bromoketone functionality into hydrolytically stable β-*C*-glycosides.

Of interest as targets for these novel β-*C*-glycosides are β-glucosidases and endo and exo-glucanases. *Agrobacterium* sp. β-glucosidase has undergone rigorous mechanistic investigations, but attempts to selectively label the acid/base catalyst have so far been unsuccessful. In addition, β-(1,4)-glucanases from *C. fimi* are attractive targets for these compounds since the acid/base catalysts in most of these enzymes have not been identified by labeling experiments. Although the acid/base catalyst in the exoglucanase/xylanase, Cex, was labeled and identified using *N*-bromoacetyl-β-D-cellobiosylamine, other glucanases, such as CenD, are not inactivated by this compound. New compounds are therefore required to probe the wide range of cellulases and their different specificities.

Results and Discussion

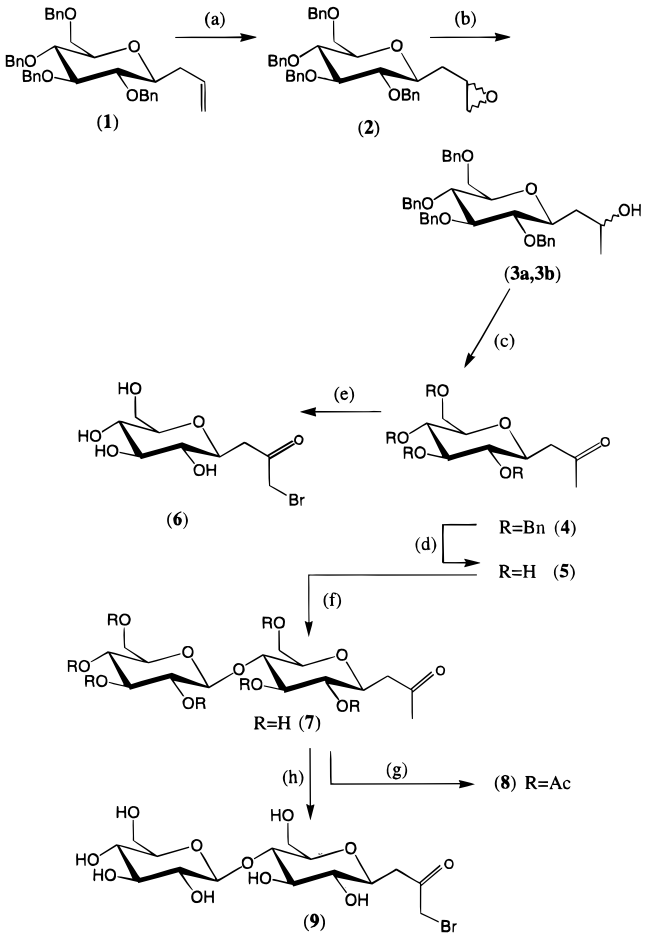
The synthetic strategy was based on β-*C*-glycoside ketones **5** and **7** which were obtained from the allyl-β-*C*-glucoside (**1**) via oxidation of the olefin function (Scheme 1). The final bromoketones were obtained by acid-catalyzed bromination at the α-positions with respect to the ketone functionalities.

Allyl-β-*C*-glucoside (**1**) was readily prepared from Grignard addition to benzylated 1,5-gluconolactone.[23] A one-step conversion of this compound to the ketone **4** with a Wacker oxidation was unsuccessful due to the low solubility of the starting material under the reaction conditions. Epoxidation of the olefin **1** with mCPBA followed by reduction with lithium bis(diisopropylbutyl) aluminum hydride[24] (prepared in situ) gave the diastereomeric alcohols **3a**,**b**. Both diastereomers were then converted to the ketone **4** via the Jones' oxidation. Deprotection and bromination of **4** gave the desired material **6**.

Best et al. have described the synthesis of allyl-β-*C*-cellobioside in an analogous fashion to **1**, but it was reported that Grignard additions to β-D-cellobionolactone are low yielding.[25] As an alternative route to the target material **9**, the β-*C*-cellobioside **7** was synthesized via enzymatic addition of a second glucose unit to the β-*C*-glycoside **5**. This was achieved by incubating the Glu358Ala mutant of *Agrobacterium* sp. β-glucosidase[26] with the glycosyl acceptor **5** and α-glucosyl fluoride as the glucosyl donor, at concentrations of 100 and 50 mM, respectively. The desired cellobioside **7** was obtained in 21% yield (based on recovered starting material) with lesser amounts of trisaccharide products. Repeating the reaction with a higher concentration of **5** (150 mM), double the buffer concentration (200 mM), and purification by flash chromatography rather than HPLC provided a small improvement in the yield (25%). The relatively low yields for this step are a reflection of the strong preference of this enzyme for an aromatic aglycon.[27] Acetylation of the cellobioside **7** and analysis of the ^{1}H NMR-COSY spectrum revealed an unambiguous β(1,4)-linkage. Bromination of **7** gave the desired product **9**.

The β-*C*-glucoside **6** caused time-dependent inactivation of *Agrobacterium* sp. β-glucosidase according to first-order kinetics. Residual enzyme activity, at several different concentrations of **6**, was measured over time, and the observed pseudo-first-order rate constants for inactivation were determined for each

Scheme 1. Synthesis of β-*C*-Glucoside (**6**) and β-*C*-Cellobioside (**9**)[a]



[a] (a) mCPBA, CH_2Cl_2, 85%; (b) DIBAL/BuLi, THF, 92%; (c) CrO_3, H_2SO_4, 86%; (d) 5% Pd−C, H_2, 10% AcOH−MeOH, 90%; (e) **5**, MeOH, Br_2, 53%; (f) *Agrobacterium* sp. β-glucosidase-E358A mutant, β-glucosyl fluoride, pH 7.0, 21%; (g) pyridine/Ac_2O, 33%; (h) MeOH, Br_2, 58%.

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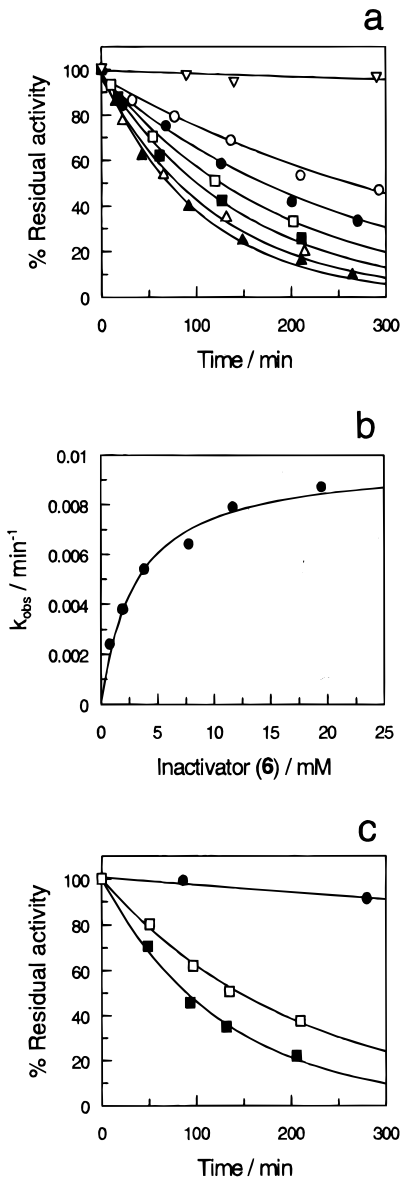
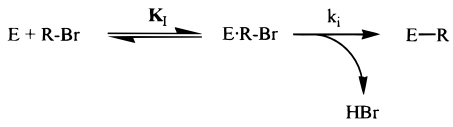


Figure 1. Inactivation of *Agrobacterium* sp. β-glucosidase by **6**. (a) Plot of residual activity versus time at the following inactivator concentrations: ▽, 0 mM; ○, 0.78 mM; ●, 1.95 mM; □, 3.9 mM; ■, 7.8 mM; △, 11.7 mM; ▲, 19.5 mM (activity ∝ rate of β-PNPG hydrolysis). (b) Plot of pseudo-first-order rate constants from panel a versus inactivator concentration. (c) Plot of residual activity versus time in the presence of (●) no inactivator, (□) 7.6 mM **6** + 15 mM IPTG, and (■) 7.6 mM **6**.

Scheme 2



concentration (Figure 1a). A plot of these rate constants versus inactivator concentration is shown in Figure 1b. The saturation kinetic behavior observed is compatible with the kinetic model shown in Scheme 2.

Values of $K_I = 3.1$ (±0.53) mM and $k_i = 0.01$ (±0.0005) min^{-1} were determined directly from this plot. Active-site-directed protection against inactivation was demonstrated by the use of 15 mM IPTG, the first-order rate constant for inactivation by **6** (7.6 mM) being reduced from 0.0077 to 0.0048 min^{-1} (Figure 1c). ESMS analysis of the inactive enzyme in the LC/MS mode (data not shown) revealed three major species corresponding to protein molecules having 11, 12, and 13 labels attached, a result very similar to that obtained with this enzyme using *N*-bromoacetyl-β-D-glucosylamine.[21]

When the β-*C*-cellobioside analogue **9** was tested as an inactivator of six different cellulases, its inactivation behavior was found to be profoundly different from that with *N*-bromoacetyl-β-D-cellobiosylamine. Whereas *N*-bromoacetyl-β-D-cellobiosylamine is an effective inactivator of the exoglucanase (Cex),[19] **9** provided no inactivation. Further, both **6** and **9** were found to inactivate the endoglucanase (CenA), yet only **9** was able to inactivate CenD. Both CenA and CenD are readily assayed with the chromogenic substrate β-DNPC. These were therefore subjected to kinetic analysis in the presence of **9** using methods analogous to those described above for *Agrobacterium* sp. β-glucosidase.

The β-*C*-cellobioside **9** caused time-dependent inactivation of CenD according to pseudo-first-order kinetics (Figure 2a). Data were not recorded beyond 600 min due to indications from TLC that **9** begins to decompose after this length of time. Values of $K_I = 6.0$ (±0.9) mM and $k_i = 0.01$ (±0.001) min^{-1} were determined by fitting the data to Scheme 2 using a nonlinear regression analysis (Figure 2b). Active-site-directed protection was afforded by 50 mM cellobiose, the pseudo-first-order rate constant for enzyme inactivation being reduced from 0.0053 to 0.0011 min^{-1} in the presence of 6.04 mM (**9**). ESMS analysis of CenD using the spectrometer in the LC/MS mode revealed one major species of 75 016 (±8) Da corresponding to the native enzyme (Figure 3a). The same analysis of a partially inactivated sample of CenD [**9** (7 mM), 22 °C, 5 h] showed two species, 75 021 (±8) Da and 75 394 (±9) Da, which correspond to the unlabeled and labeled enzyme, respectively (Figure 3b). The mass increase of 373 Da is consistent with the expected mass increase of 381 Da as a result of the stoichiometric labeling of CenD with one molecule of **9**. However, incubation of CenD with **9** for extended periods of time (15 h) resulted in the addition of a second label. This is not surprising given the presence of the inherently reactive bromoketone functionality, and also given that the enzyme must contain an extended oligosaccharide binding site into which the label may bind.

CenA was also inactivated by **9** in a time-dependent manner (Figure 4a), values of $K_I = 0.35$ (±0.02) mM and $k_i = 0.0155$ (±0.0004) min^{-1} being determined directly from a plot of k_{obs} versus inactivator concentration (Figure 4b). Again, cellobiose (50 mM) was shown to afford protection from inactivation, reducing the pseudo-first-order rate constant for enzyme inactivation by **9** (0.51 mM) from 0.0093 min^{-1} to essentially zero (Figure 4c). LC/MS analysis of CenA was conducted in an analogous fashion to that described for CenD. Analysis of unlabeled CenA (catalytic domain only) revealed a pair of peaks of masses 43 829 (±5) and 44 102 (±5) Da (Figure 5a), the different masses corresponding to slightly different positions of cleavage when the cellulose binding domain was removed proteolytically. Incubation with **9** (7 mM, 22 °C, 5 h) followed by ESMS analysis revealed that both species were labeled to an extent of approximately 70% as evidenced by the appearance of two new species of masses 44 211 (±5) and 44 480 (±5) Da (Figure 5b). The mass increase of 380 Da is again consistent with stoichiometric labeling and, as with CenD, prolonged incubation (15 h) resulted in some secondary labeling.

The endocellulase, CenB, and the cellobiosyl hydrolases, CbhA and CbhB, do not hydrolyze arylglycosides and so cannot be assayed as readily. In addition to making assays more

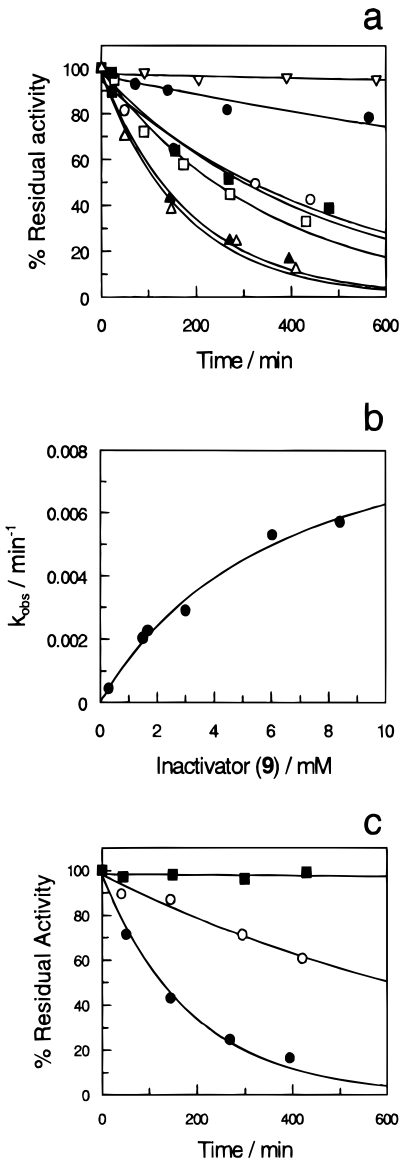


Figure 2. Inactivation of CenD by **9**. (a) Plot of residual activity versus time at the following inactivator concentrations: ▽, 0 mM; ●, 0.3 mM; ○, 1.51 mM; ■, 1.68 mM; □, 3.02 mM; ▲, 6.04 mM; △, 8.4 mM (activity ∝ rate of β-DNPC hydrolysis). (b) Plot of pseudo-first-order rate constants from panel a versus inactivator concentration. (c) Plot of residual activity versus time in the presence of (■) no inactivator, (○) 6.04 mM **9** + 50 mM cellobiose, and (●) 6.04 mM **9**.

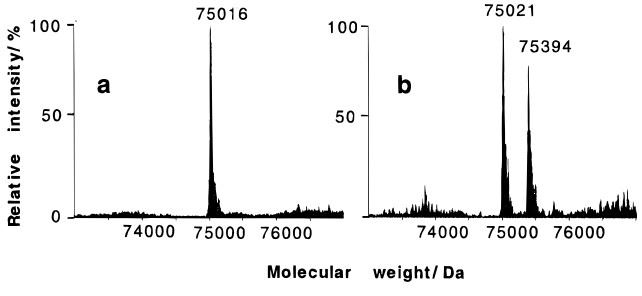


Figure 3. Electrospray mass spectrometry of CenD. (a) Reconstructed mass spectrum of unlabeled enzyme. (b) Reconstructed mass spectrum of enzyme partially inactivated by **9**.

difficult, this also made it less likely that the affinity labels **6** or **9** would bind to and inactivate these enzymes. Nonetheless, the possibility of labeling was monitored by ESMS. However, after incubation with **9** for 15 h, no labeling was observed.

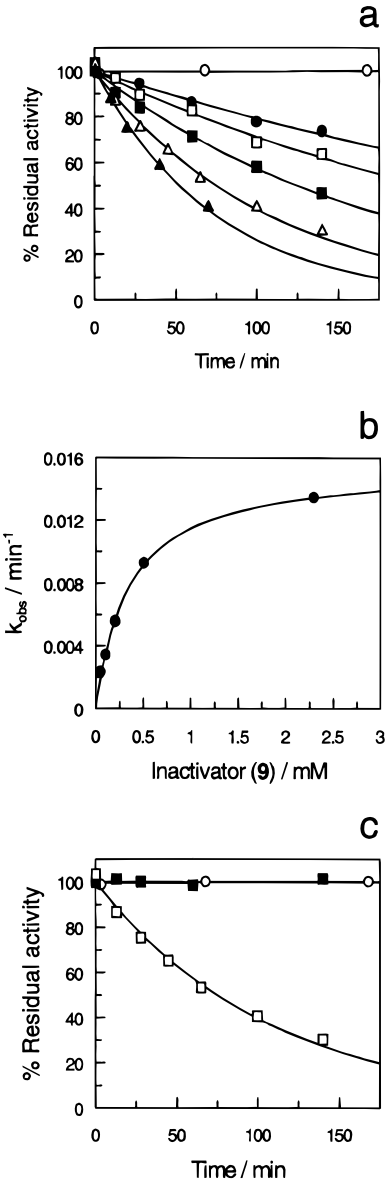


Figure 4. Inactivation of CenA by **9**. (a) Plot of residual activity versus time at the following inactivator concentrations: ○, 0 mM; ●, 0.05 mM; □, 0.10 mM; ■, 0.20 mM; △, 0.51 mM; ▲, 2.3 mM (activity ∝ rate of β-DNPC hydrolysis). (b) Plot of pseudo-first-order rate constants from panel a versus inactivator concentration. (c) Plot of residual activity versus time in the presence of (○) no inactivator, (■) 0.51 mM **9** + 50 mM cellobiose, and (□) 0.51 mM **9**.

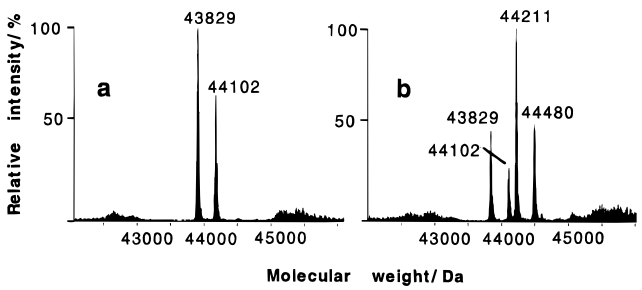


Figure 5. Electrospray mass spectrometry of CenA. (a) Reconstructed mass spectrum of unlabeled enzyme. (b) Reconstructed mass spectrum of enzyme partially inactivated by **9**.

Clearly, there are profound differences between the reactivity of these new affinity labels **6** and **9** and their *N*-(bromoacetyl)-glycosylamine counterparts discussed earlier. What was not clear a priori was whether these differences are the result of

differences in inherent reactivity or a consequence of different binding modes of these compounds in the enzyme active site leading to different placement of the reactive functionalities with respect to active site amino acids. In general α-halo esters and amides tend to be less reactive than the corresponding α-haloketones. However, the relative reactivities depend considerably upon the nucleophile involved; thus, it is not easy to predict which will react faster when the identity of the nucleophile is unknown.[28,29] In one case (*Agrobacterium* sp. *β*-glucosidase), the bromoketone **6** and α-bromoamide exhibit very similar inactivation behaviors. By contrast, only the α-bromoamide is capable of inactivating Cex, the bromoketone having no effect. It would therefore seem unlikely that the inherent reactivity of the inhibitor is the dominant factor, but rather the different binding modes likely mediated via hydrogen bonding to the amide moiety.

It is also of interest to note that the second-order rate constants for inactivation ($k_i/K_i = 3.2$, 1.7, and 44.3 $M^{-1}min^{-1}$ for CenD, Abg, and CenA, respectively) are several orders of magnitude greater than those measured previously for reaction of acetate anion with bromoketones. For example, bromomethyl neopentyl ketone, a similarly substituted derivative, reacts with acetate anion at 54 °C with a second-order rate constant of 5.5×10^{-2} M^{-1} min^{-1}.[30] Reactions with acetic acid rather than acetate are typically much slower still.[31] The higher reaction rates at the enzyme active site presumably reflect the location of the carboxylic nucleophile in close proximity to the reactive center.

Conclusion

These α-bromoketones therefore constitute a valuable new class of affinity labels for glycosidases in general, and cellulases in particular. The reactivity of this functionality is sufficiently different from that of the known α-bromoamides and exocyclic epoxides that they can be used in a complementary manner. Further, the ability to synthesize α-bromoketones with the α-anomeric configuration, an extremely difficult task for the α-bromoamides, opens up the possibility of using this approach with α-glycosidases. In addition to simply providing reagents for the inactivation of the enzymes in question, they should be valuable tools for the identification of the residues so tagged through mass spectrometric analysis of proteolytic digests of labeled enzymes.

Experimental Methods

Synthesis. All buffer chemicals and other reagents were obtained from the Sigma/Aldrich Chemical Co. unless otherwise noted. Reactions were monitored by TLC using Merck Kieselgel 60 F_{254} aluminum-backed sheets. Compounds were detected by charring with 10% ammonium molybdate in 2 M H_2SO_4 and heating. Flash chromatography under a positive pressure was performed with Merck Kieselgel 60 (230−400 mesh) using the specified eluents. ^{1}H NMR spectra were recorded on a Brüker WH-400 spectrometer at 400 MHz (chemical shifts quoted relative to $CDCl_3$ or DSS when taken in D_2O). ^{13}C NMR spectra were recorded on a Varian XL-300 at 75 MHz or a Brüker AC-200 at 50 MHz and are proton-decoupled with $CDCl_3$ or acetone (30.5 ppm) as a reference.

1′,2′-Epoxy-3′-(2,3,4,6-tetra-*O*-benzyl-*β*-D-glucopyranosyl)propane (2). 3′-(2,3,4,6-Tetra-*O*-benzyl-*β*-D-glucopyranosyl)-1′-propene (**1**) (2.39 g, 4.24 mmol), *m*-chloroperbenzoic acid (80%, 1.79 g, 8.32 mmol), and 2,6-di-*tert*-butyl-4-methylphenol (20 mg) were dissolved in dichloromethane (60 mL), and the mixture was stirred at reflux for 4 h. After being cooled to room temperature, the mixture was diluted with dichloromethane (60 mL) and washed successively with aqueous 10% sodium metabisulfite (50 mL), 10% aqueous $NaHCO_3$ (3 × 50 mL), and saturated brine (50 mL). The organic layer was dried ($MgSO_4$) and filtered and the residue purified on silica gel (ethyl acetate: petroleum ether (bp 35−60 °C), 1:6) to give the epoxide (**2**) (1.92, 3.40 mmol, 85%) as a colorless solid (mixture of diastereoisomers). $[\alpha]_D$: +1.2 ° (*c* 0.92, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.13−7.38 (m, Ar), 4.79−4.95 (m), 4.51−4.69 (m), 3.58−3.79 (m), 3.38−3.40 (m), 3.26 (t, J 9.0 Hz), 3.07−3.16 (m), 2.74 (dt, J 15.3 and 4.5 Hz), 2.47−2.50 (m), 1.83−2.0 (m), 1.63 (ddd, J 14.1, 9.8 and 4.8 Hz, H-3′).

2′-Hydroxy-3′-(2,3,4,6-tetra-*O*-benzyl-*β*-D-glucopyranosyl)propane (3a,b). Diisobutylaluminum hydride (1 M in hexane, 20 mL, 20 mmol) was dissolved in tetrahydrofuran (50 mL) and cooled to 0 °C, and then *n*-butyllithium (1.6 M in hexane, 12.5 mL, 20 mmol) was added dropwise. After being stirred for 30 min, the solution was added to a stirred solution of the epoxide (**2**) (1.92 g, 3.40 mmol) in tetrahydrofuran (50 mL) at 0 °C.

After 2 h, the reaction was quenched with ice-cold water (200 mL) and then acidified with aqueous HCl (0.5 M). The aqueous mixture was extracted with diethyl ether (3 × 200 mL), and the combined organic fractions were washed successively with 10% aqueous $NaHCO_3$ (200 mL) and saturated brine (200 mL). The organic layer was dried ($MgSO_4$) and filtered and the solvent removed in vacuo. Purification on silica gel (ethyl acetate:petroleum ether (bp 35−60 °C), 1:3) gave a 1:1 mixture of **3a** and **3b** (1.77 g, 3.05 mmol, 92%). Several of the fractions contained pure samples of each diastereoisomer.

3a. Mp: 107−108 °C (ethyl acetate/petroleum ether, needles). $[\alpha]_D$: −4.5° (*c* 1.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.18−7.39 (20 H, m, OBn), 4.78−4.93 (4 H, m, CH_2Ar), 4.47−4.64 (4 H, m, CH_2Ar), 4.02 (1 H, m, H-2′), 3.54−3.72 (5 H, m), 3.42 (1 H, dt, *J* 9.1 and 3.1 Hz), 3.37 (1 H, t, *J* 9.0 Hz), 2.09 (1 H, br, OH), 1.85 (1 H, ddd, $J_{3a',3b'}$ 14.5 Hz, *J* 8.5 and 3.2 Hz, H-3a′), 1.71 (1 H, ddd, *J* 14.5, 7.3, and 2.4 Hz, H-3b′), 1.14 (3 H, d, $J_{1',2'}$ 7.0 Hz, H-1′). Anal. Calcd for $C_{37}H_{42}O_6$: C, 76.26; H, 7.26. Found: C, 76.47; H. 7.10. CIMS (NH_3): *m*/*z* 600 $(M + NH_4)^+$ (100%).

3b. Mp: 116−117 °C (ethyl acetate/petroleum ether, needles). $[\alpha]_D$: +1.7° (*c* 1.2, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.15−7.38 (20 H, m, Ar), 4.77−4.89 (4 H, m, CH_2Ar), 4.48−4.63 (4 H, m, CH_2Ar), 3.97−4.03 (1 H, m, H-2′), 3.62−3.69 (2 H, m), 3.45−3.55 (4 H, m), 3.25 (1 H, t, *J* 9.1 Hz), 1.91 (1 H, d, $J_{3a'3b'}$ 14.4 Hz, H-3a′), 1.57 (1 H, br, OH), 1.42 (1 H, dt, $J_{3b',2'}$ and $J_{3b',1'}$ 9.9 Hz, H-3b′), 1.14 (3 H, d, $J_{1',2'}$ 6.2 Hz, H-1′). Anal. Calcd for $C_{37}H_{42}O_6$: C, 76.26; H, 7.26. Found: C, 76.42; H. 7.19.

3′-(2,3,4,6-Tetra-*O*-benzyl-*β*-D-glucopyranosyl)-2′-propanone (4). A mixture of alcohols **3a**,**b** (630 mg, 1.08 mmol) was dissolved in acetone (10 mL), and then Jones reagent (15 g of CrO_3 100 mL of H_2O, 24 g of concentrated sulfuric acid) (1.5 mL) was added while being stirred at 0 °C. After 1 h, excess reagent was reduced using a minimum amount of 5% sodium metabisulfite and then the mixture diluted with water (50 mL). The aqueous mixture was extracted with diethyl ether (3 × 50 mL), and the combined organic fractions were washed successively with 10% aqueous $NaHCO_3$ (50 mL) and saturated brine (50 mL). The organic layer was dried ($MgSO_4$) and filtered and the solvent removed in vacuo. Purification on silica gel (diethyl ether: petroleum ether (bp 35−60 °C), 2:3) gave the ketone (**4**) (540 mg, 0.93 mmol, 86%) as a crystalline solid. Mp: 72−73 °C (diethyl ether/petroleum ether, needles). $[\alpha]_D$: −3° (*c* 1.4, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.16−7.40 (m, Ar), 4.80−4.95 (4 H, m, CH_2Ar), 4.48−4.64 (4 H, m, CH_2Ar), 3.78 (1 H, td, $J_{1,2}$ 9 Hz, $J_{1,3b'}$ 9.0 Hz, $J_{1,3a'}$ 3.5 Hz, H-1), 3.63−3.72 (4 H, m), 3.44 (1 H, dt, $J_{5,4}$ 9.4 Hz, $J_{5,6a}$ and $J_{5,6b}$ 2.9 Hz, H-5), 3.31 (1 H, t, *J* 9.1 Hz), 2.71 (1 H, dd, $J_{3a',3b'}$ 15.6 Hz, H-3a′), 2.56 (1 H, dd, H-3b′), 2.13 (3 H, s, Me). ^{13}C NMR (50 MHz, $CDCl_3$): *δ* 205.5 (C-2′), 138.3 (Ar), 128−130 (Ar), 86.9, 82.5, 78.5, 79.0, 75.5 (5 × CH), 75.0, 74.5, 73.5, 69.5 (5 × CH_2, two signals coincide), 45.5 (C-3′), 21.0 (C-1′). Anal. Calcd for $C_{37}H_{40}O_6$: C, 76.53; H, 6.94. Found: C, 76.50; H, 6.86. CIMS (NH_3): *m*/*z* 598 $(M + NH_4)^+$(40%), 581 $(M + H)^+$ (20%).

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3′-(β-D-Glucopyranosyl)-2′-propanone (5). Ketone **4** (540 mg, 0.93 mmol) was dissolved in 10% acetic acid−methanol (20 mL) and stirred with 5% Pd−C (10 mg) under hydrogen at room temperature and pressure. After 16 h, the mixture was filtered, the solvent removed in vacuo, and the residue purified on silica gel (ethyl acetate:methanol: water, 15:4:1) to give **5** (180 mg, 0.84 mmol, 90%) as a colorless syrup. $[\alpha]_D$: −3° (*c* 1.3, MeOH). ^{1}H NMR (400 MHz, D_2O): δ 3.82 (1 H, dd, $J_{6a,6b}$ 12.2 Hz, $J_{6a,5}$ 1.8 Hz, H-6a), 3.75 (1 H, td, $J_{1,2}$ and $J_{1,3b'}$ 9.5 Hz, $J_{1,3a'}$ 3.1 Hz, H-1), 3.64 (1 H, dd, $J_{6b,5}$ 5.1 Hz, H-6b), 3.45 (1 H, t, 8.7 Hz), 3.31−3.41 (2 H, m), 3.19 (1 H, t, J 9.3 Hz), 2.98 (1 H, dd, $J_{3a',3b'}$ 16.6 Hz, H-3a′), 2.68 (1 H, dd, H-3b′), 2.24 (3 H, Me). CIMS (NH_3): *m*/*z* 221 $(M + H)^+$ (100%), 238 $(M + NH_4)^+$(30%). HRMS: $(M + H)^+$, Calcd 221.1020, found 221.1030.

1′-Bromo-3′-(β-D-glucopyranosyl)-2′-propanone (6). Ketone **5** (150 mg, 0.66 mmol) was dissolved in methanol (2 mL), to which was added bromine (53 μL, 0.99 mmol) as a solution in methanol (0.5 mL). After 3 h at 40 °C, the solvent was removed in vacuo and the residue purified on silica gel (ethyl acetate:methanol:water, 17:3:1) to give the α-bromoketone (**6**) (106 mg, 0.35 mmol, 53%) as a foamy solid. ^{1}H NMR (400 MHz, D_2O) (assignments confirmed by COSY): δ 4.33 (2 H, s, H-1′), 3.81 (1 H, dd, $J_{6a,6b}$ 12.4 Hz, $J_{6a.5}$ 1.6 Hz, H-6a), 3.77 (1 H, td, $J_{1,2}$ and $J_{1,3b'}$ 9.0 Hz, $J_{1,3a'}$ 3.4 Hz, H-1), 3.64 (1 H, dd, $J_{6b,5}$ 5.1 Hz, H-6b), 4.35 (1 H, t, $J_{3,2}$ and $J_{3,4}$ 9.0 Hz, H-3), 3.33−3.48 (2 H, m, H-5 and H-4), 3.21 (1 H, t, H-2), 3.11 (1 H, dd, $J_{3a',3b'}$ 16.5 Hz, H-3a′), 2.87 (1 H, dd, H-3b′). ^{13}C NMR (50 MHz, D_2O): δ 204.5 (C-2), 70.2, 73.5, 75.8, 77.6, 80.0 (5 × CH), 61.2 (C-6), 36.9, 42.9 (2 × CH_2). CIMS (NH_3): *m*/*z* 316 $(M + NH_4)^+$ (25%), 318 $(M + NH_4)^+$ (25%), 299 $(M + H)^+$ (10%), 301 $(M + H)^+$ (10%). Anal. Calcd for $C_9H_{15}O_6Br$: C, 36.14; H, 5.05. Found: C, 36.07; H, 5.24.

3′-(β-D-Cellobiosyl)-2′-propanone (7). β-Glucosidase (E358A mutant from *Agrobacterium* sp.) (0.96 mg) was incubated with 3′-(β-D-glucopyranosyl)-2′-propanone (**5**) (150 mg, 0.682 mmol) and α-D-glucosyl fluoride (62 mg, 0.34 mmol) in sodium phosphate buffer (150 mM, pH 7.0). After 24 h, the enzyme was reclaimed by dialysis using a centrifugal ultrafilter with a molecular weight cutoff of 10 000 (Millipore). The filtrate was evaporated to dryness in vacuo and the residue suspended in methanol (4 mL). The methanolic solution was filtered through a silica plug which was further washed with methanol (20 mL). The solvent was removed in vacuo, and the residue was dissolved in methanol (4 mL) and then filtered (2.2 μm filter). The solvent was removed in vacuo and the residue purified on a Dynamax reverse phase column using a Waters HPLC system and eluting with 70% acetonitrile in water. Starting material **5** (84 mg, 0.38 mmol) and β-*C*-cellobioside **7** (24 mg, 0.063 mmol, 21% based on recovered staring material) were obtained as colorless syrups. Material with a longer retention time, presumably trisaccharides, was also recovered (15 mg). β-*C*-Cellobioside **7**: $[\alpha]_D$: +1.9° (*c* 1.0, MeOH). ^{1}H NMR (400 MHz, D_2O): δ 4.46 (1 H, d, $J_{1'',2''}$ 8.0 Hz, H-1″), 3.84−3.91 (2 H, m), 3,66−3.79 (3 H, m), 3.55−3.60 (2 H, m), 3.35−3.59 (4 H, m), 3.21−3.29 (2 H, m). 2.98 (1 H, dd, $J_{3a',3b'}$ 17.7 Hz, $J_{3a',1}$ 2.5 Hz, H-3a′), 2.69 (1 H, dd, $J_{3b',1}$ 9.2 Hz, H-3b′), 2.23 (3 H, s, Me). ^{13}C NMR/APT (75 MHz, D_2O): δ 213.9 (C-2′), 103.3 (C-1″), 70.2, 73.6, 73.9, 75.8, 76.2, 76.4, 76.7, 79.1, 79.5 (9 × CH), 60.8, 61.3 (C-6 and C-6″), 46.3 (C-3′), 31.0 (C-1′). CIMS (NH_3): *m*/*z* 400 $(M + NH_4)^+$ (100%). HRMS: $(M + NH_4)^+$ calcd 400.1810, found: 400.1837.

3′-(2,3,6,2″,3″,4″,6″-Hepta-*O*-acetyl-β-D-cellobiosyl)-2′-propanone (8). A crude sample of the β-*C*-cellobioside **7** (22 mg, 0.1 mmol) was dissolved in pyridine (1.5 mL) and acetic anhydride (0.5 mL) and allowed to stand for 16 h. The solvent was removed in vacuo and the residue purified on silica gel (ethyl acetate:petroleum ether (bp 35−60 °C), 2:1) to give the acetylated β-*C*-cellobioside **8** (13 mg, 0.015 mmol, 33%) as a colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$, assignments confirmed by COSY): δ 5.14 (1 H, t, $J_{3,2}$ and $J_{3,4}$ 9.5 Hz, H-3), 5.11 (1 H, t, $J_{3'',2''}$ and $J_{3'',4''}$ 9.7 Hz, H-3″), 5.04 (1 H, t, $J_{4'',5''}$ 9.6 Hz, H-4″), 4.89 (1 H, dd, $J_{2'',1''}$ 8.0 Hz, H-2″), 4.79 (1 H, t, $J_{2,1}$ 9.5 Hz, H-2), 4.48 (1 H, d, H-1″), 4.42 (1 H, dd, $J_{6a,6b}$ 12.0 Hz, $J_{6a,5}$ 1.8 Hz, H-6a), 4.34 (1 H, dd, $J_{6a'',6b''}$ 12.4 Hz, $J_{6a'',5''}$ 4.4 Hz, H-6a″), 4.06 (1 H, dd, $J_{6b,5}$ 5.3 Hz, H-6b), 4.03 (1 H, dd, $J_{6b'',5''}$ 2.1 Hz, H-6b″), 3.89 (1 H, td, $J_{1,3a'}$ 9.5 Hz, $J_{1,3b'}$ 3.1 Hz, H-1), 3.70 (1 H, t, $J_{4,5}$ 9.5 Hz, H-4), 3.63 (1 H, ddd, H-5″), 3.57 (1 H, ddd, H-5), 2.63 (1 H, dd, $J_{3a',3b'}$ 16.3 Hz, H-3a′), 2.42 (1 H, dd, H-3b′), 2.13, 2.07, 2.06, 2.0, 1.99, 1.95 (18 H, 6 × s, 6 × Me), 1.98 (6H, s). CIMS (NH_3): *m*/*z* 694 $(M + NH_4)^+$ (35%), 677 $(M + H)^+$. HRMS: $(M + H)^+$ calcd 677.2280, found 677.2291.

1′-Bromo-3′-(β-D-cellobiosyl)-2′-propanone (9). The β-*C*-cellobioside **7** (20 mg, 0.052 mmol) and bromine (5 μL, 0.094 mmol) were dissolved in methanol (0.5 mL) and allowed to stand at 40 °C for 1 h. The solvent was removed in vacuo and the residue purified on silica gel (ethyl acetate:methanol:water, 10:3:1) to give 1′-bromo-3′-(β-D-cellobiosyl)-2′-propanone (**9**) (14 mg, 58%) as a colorless syrup. $[\alpha]_D$: −10.9° (*c* 0.95, MeOH). ^{1}H NMR (400 MHz, D_2O): δ 4.46 (1 H, d, $J_{1'',2''}$ 7.9 Hz, H-1″), 4.31 (2 H, s, CH_2Br), 3.83−3.91 (2 H, m), 3.66−3.80 (3 H, m), 3.55−3.61 (2 H, m), 3.43−3.53 (3 H, m), 3.34−3.40 (1 H, m), 3.23−3.31 (2 H, m), 3.11 (1 H, dd, $J_{3a',3b'}$ 16.5 Hz, $J_{3a',1}$ 3.1 Hz, H-3a′), 2.87 (1 H, dd, $J_{3b',1}$ 8.9 Hz, H-3b′). ^{13}C NMR/APT (75 MHz, D_2O): δ 204.2 (C-2), 102.7 (C-1″), 69.7, 73.1, 73.4, 75.5, 75.7, 75.9, 76.2, 78.5, 78.9 (9 × CH), 60.2, 60.8 (C-6 and C-6″), 42.6 (C-1′), 36.7 (C-3′). ESMS: *m*/*z* 483.5 $(M + Na)^+$ (100%), 485 $(M + Na)^+$ (100%).

Enzyme Kinetics. Recombinant *Agrobacterium* sp. β-glucosidase was purified as described previously.[30] Recombinant CenA, CenD, and Cex were generously donated by Dr. Neil Gilkes from the Department of Microbiology, University of British Columbia, and were purified as described previously.[1−4,7,32] A continuous spectrophotometric assay based on the hydrolysis of nitrophenyl glycosides was used to monitor enzyme activity by measurement of the rate of nitrophenolate release using a UNICAM 8700 UV−visible spectrophotometer equipped with a circulating water bath.

Inactivation of enzymes by **6** or **9** was monitored by incubation of the enzyme with various concentrations of the inactivator at 37 °C. Residual enzyme activity was determined at the appropriate time intervals by addition of a 10-μL aliquot of the inactivation mixture to a solution of substrate and measurement of the corresponding nitrophenolate release at 37 °C to an extent no greater than 10% substrate depletion. The same buffer conditions were used for both the inactivation mixtures and assays. Individual conditions were as follows: *Agrobacterium* sp. β-glucosidase, 50 mM sodium phosphate, pH 6.8, 0.1% BSA, assay 800 μL × 1 mM β-PNPG (K_m = 0.1 mM); CenA and CenD, 50 mM sodium phosphate, pH 7.0, 0.1% BSA, assay 200 μL × 2 mM β-DNPC ($K_{m[CenA]}$ = 0.17 mM[1], $K_{m[CenD]}$ = 0.56 mM]); Cex, same as for CenA/CenD except assayed using 800 μL × 6 mM β-PNPC (K_m = 0.6 mM). Pseudo-first-order inactivation rate constants at each inactivator concentration (k_{obs}) were determined by fitting each curve to a first-order equation using the program GraFit (Leatherbarrow, R. J. *GraFit Version 3.0*; Erithacus Software Ltd.: Staines, U.K., 1990). The values of k_i and K_I, assuming inactivation according to the kinetic model shown in Scheme 2, were determined from a direct fit to the plot of k_{obs} against inactivator concentration.

Electrospray Mass Spectrometry. The analyses of the protein samples were carried out using a Sciex API-300 mass spectrometer interfaced with a Michrom UMA HPLC system (Michrom Bioresources, Inc., Auburn, CA). Enzymes were incubated for 5 h (22 °C) under the following conditions prior to LC/MS analysis: *Agrobacterium* sp. β-glucosidase, 19.5 mM **6**; CenA and CenD, 7 mM **9**. The enzyme (10−20 μg, labeled or unlabeled) was introduced into the mass spectrometer through a microbore PLRP column (1 × 50 mm) and eluted with a gradient of 20−100% solvent B at a flow rate of 50 μL/min over 15 min (solvent A: 0.06% trifluoroacetic acid, 2% acetonitrile in water; solvent B: 0.05% trifluoroacetic acid, 90% acetonitrile in water). The MS was scanned over a range of 400−2300 Da with a step size of 0.5 Da and a dwell time of 1 ms.

Acknowledgment. We thank Karen Rupitz for technical assistance, Lloyd Mackenzie for advice, and Dr. Neil Gilkes for providing enzyme samples and valuable discussion. We thank the Protein Engineering Network of Centres of Excellence of Canada and the Natural Sciences and Engineering Research Council of Canada for financial support.

JA981580R

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