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Note

Affinity labelling of *Schizophyllum commune* cellulase with [1-^{3}H]-4,5-epoxypentyl β-cellobioside: Synthesis of inhibitor and stoichiometry of interaction*

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The use of epoxyalkyl glycosides as active-site-directed inhibitors has been invaluable in delineating the mechanism of action for a variety of carbohydrases, including lysozyme[1,2], β-D-glucosidase[3], beta amylase[4], and cellulase[5–7]. Detailed studies with this class of compounds have revealed that the length of the aglycon chain is critical for optimum rates of inactivation. Stone and co-workers[7] found that, of a series of epoxide derivatives tested, 3,4-epoxybutyl cellobioside served as the most efficient affinity label for the β-D-glucanase from *Bacillus subtilis*. On the other hand, 4,5-epoxypentyl cellobioside proved to be the most potent active-site-directed inactivator of the fungal cellulases isolated from an *Oxyporum* and two *Aspergillus* species[5]. We have successfully employed the latter reagent to affinity-label the cellulase from the white-rot fungus, *Schizophyllum commune*[6]. However, the lack of radiolabelled derivatives of these cellulase inhibitors seriously impairs their usefulness for probing the active sites of various enzymes of interest. Indeed, only indirect approaches, involving either displacement of the covalently bound inhibitor[6] or molecular-weight determinations of the inactivated cellulase[7], were used to determine the stoichiometry of the reaction of these affinity labels with the respective enzymes. We have thus undertaken the synthesis of [1-^{3}H]-4,5-epoxypentyl β-cellobioside, and report here on the direct determination of the stoichiometry of the affinity label–cellulase interaction.

The synthesis of [1-^{3}H]-4,5-epoxypentyl β-cellobioside (**7**) was achieved by Koenigs–Knorr glycosylation of [1-^{3}H]-4-penten-1-ol (**3**). Since neither cellobiose (and any of its blocked derivatives) nor 4-penten-1-ol is commercially available in a radioactive form, that of the latter was prepared as a tritiated derivative by

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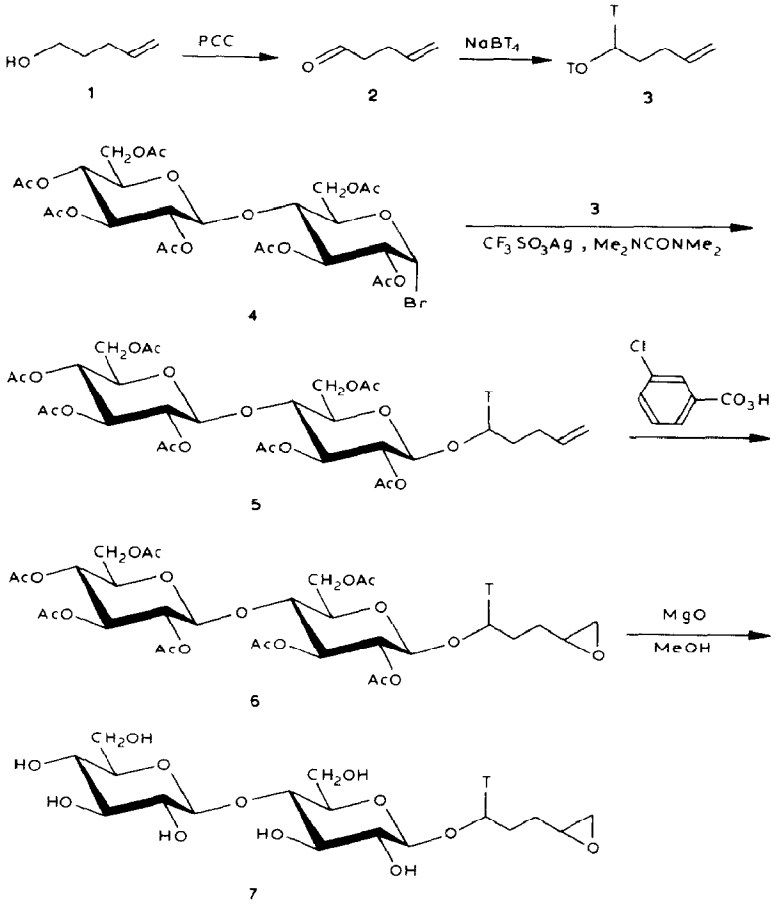
reduction of the corresponding aldehyde employing sodium boro[^{3}H]hydride. Oxidation of 4-penten-1-ol (**1**) with pyridinium chlorochromate[8] (PCC) in dichloromethane gave the volatile 4-pentenal (**2**) in 38% yield; reduction of **2** with sodium boro[^{3}H]hydride in dry ethanol gave an 84% yield of the tritiated alcohol **3** (specific activity, 0.59 Ci.mol^{-1}). The glycosylation of **3** and preparation of the tritiated affinity reagent **7** were achieved essentially as described[6] for the unlabelled compound, with one modification. Following the synthesis of [1-^{3}H]-4-pentenyl β-cellobioside heptaacetate (**5**), and its oxidation to the epoxide **6**, MgO in dry methanol[9] at 45° was used for the mild deacetylation of the blocked derivative, to give **7** in an overall yield of 14% (specific activity, 0.36 Ci.mol^{-1}). Epoxidation of the pentenyl chain of **5** introduces a new chiral center at C-4 of the aglycon. However, while the affinities of the respective diastereoisomers of **7** for the enzyme are likely to differ[10], no attempt was made to separate or purify them.



Inactivation studies had shown[6] that 4,5-epoxypentyl β-cellobioside is an active-site-directed inhibitor of the *S. commune* cellulase. In the present study, the stoichiometry of the reaction leading to the inactivation of the enzyme was investigated by incubation of the cellulase (20μM) in 50mM sodium acetate buffer, pH 5.2, with **7** (170mM). This treatment resulted in a progressive loss of the hydrolytic activity of the enzyme (see Fig. 1). Control enzyme solutions incubated in either the presence of 1,2-epoxyhexane (300mM) or the absence of added reagent retained full catalytic activity. The inactivation of the cellulase by **7** was exponential with time (see Fig. 1, inset), and, under these conditions, the enzyme lost 50% of its initial activity within ~95 min. The excess of **7** and the by-products formed during the reaction were removed by size-exclusion chromatography conducted initially on a column (0.8 × 18 cm) of Bio-Gel P6-DG (BioRad, Richmond, CA) with water serving as the eluant. Fractions containing protein were pooled and freeze-dried. Rechromatography of the pooled material on a column (0.8 × 20 cm) of Bio-Gel P60 at 4°, using 10mM ammonium acetate, pH 5.8, as the eluant, provided a homogeneous preparation of the affinity-labelled enzyme. Examination of the recovered protein fractions for the tritium label, and subsequent quantitation, revealed the presence of 0.83 mol of **7** per mol of the enzyme. These results suggested that the inactivation process is accompanied by a stoichiometric incorporation of ~1 mol of the inhibitor per mol of the enzyme. The retention of the radioactive label, even after prolonged diafiltration or gel filtration of the inactive cellulase, is indicative of a covalent modification of the enzyme. No regeneration of activity could be detected upon incubation of the inactive enzyme at pH 5.5, or on treatment with NH_2OH, pH 7.0.

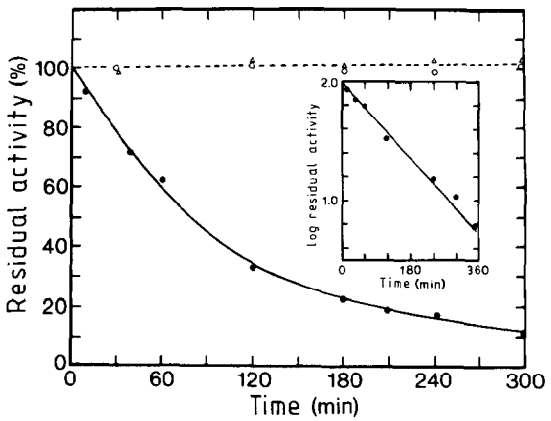


Fig. 1. Time course of inactivation of *S. commune* cellulase by compound **7**. Enzyme (20 μM) in 50mM sodium acetate buffer, pH 5.2, was incubated at 25° in the absence (○) or presence of either 170mM **7** (●) or 300mM 1,2-epoxyhexane (△). Residual activity was determined viscometrically, using 0.4% CM-cellulose in 50mM sodium acetate buffer, pH 5.0, as substrate. Inset: semilogarithmic plot of the inactivation of the cellulase by **7**. The pseudo-first-order rate-constant of the inactivation under these experimental conditions was calculated to be $\sim 1.2 \times 10^{-4}$ s^{-1}.

Amino acid sequence homology studies have led to the proposal that the *S. commune* cellulase follows the same mechanistic pathway as the hen egg-white type of lysozymes[11,12]. Two specific residues of the cellulase, Glu-33 and Asp-50, were postulated to act respectively as the general acid catalyst and the stabilizing anion. However, striking alignments with the known catalytic centers of other carbohydrases involving different regions of this cellulase are also possible[12a]. Kinetic analysis and chemical modification experiments have provided strong evidence for the participation of carboxylic acids–carboxylates in the mechanism of action of this enzyme[13]. The data presented in this study demonstrate the potential of **7** to serve as both a specific and effective affinity label for the characterization of the active site of cellulases.

EXPERIMENTAL

General methods. — Melting points were determined in a Gallenkamp apparatus and are uncorrected. Thin-layer chromatography was performed on Merck precoated silica gel 60F-254 plates, and the detection of compounds was achieved either by quenching of ultraviolet fluorescence or charring after spraying with 5% sulfuric acid in ethanol. Cellulase [endo-(1→4)-β-D-glucanase; EC 3.2.1.4] from *S. commune* was purified to homogeneity from an ethanol precipitate of the culture fluid as previously described[13]. Concentrations of the enzyme were determined either spectrophotometrically employing the molar extinction coefficient 8.53×10^4 L.mol^{-1} cm^{-1} at[14] 280 nm or by amino acid analysis assuming[12] lysine = 5 and arginine = 5. Cellulase hydrolytic activity was determined viscometrically, using a No. 1 Ubbelohde viscometer and 0.4% CM-cellulose in 50mM sodium acetate buffer, pH 5.0, as substrate. ^{13}C-N.m.r. spectra were recorded with a Bruker AM-400 spectrometer at 125 MHz and are expressed in p.p.m. relative to tetramethylsilane as an internal reference. The spectra of blocked (*O*-acetylated) derivatives were recorded for solutions in $CDCl_3$, while those of deprotected products were for solutions in D_2O.

4-Pentenal (**2**). — Following the method of Corey and Suggs[8], 4-penten-1-ol (**1**; 8.34 g, 97 mmol) was oxidized by pyridinium chlorochromate (PCC; 27.0 g, 125 mmol) in dichloromethane (175 mL). Careful fractional distillation gave the volatile **2**; yield, 3.1 g (38%); b.p. 102–105° (lit.[15] b.p. 103–104°); ^{13}C-n.m.r. data (125 MHz): δ 200.47 (C-1), 136.01 (C-4), 114.42 (C-5), 41.77 (C-2), and 25.27 (C-3).

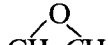
[1-^{3}H]-4-Penten-1-ol (**3**). The aldehyde **2** (0.17 g, 2.0 mmol) in 100 μL of dry ethanol was added to $NaBT_4$ (0.68 mg, 18 μmol, 25 mCi; ICN Biomedicals) and the solution allowed to stand for 18 h at 23°. The reduction was completed by the slow addition of $NaBH_4$ (0.10 g, 2.6 mmol). After standing for a further 2 h, authentic 4-penten-1-ol (0.83 g, 9.6 mmol) and water (10 mL) were added. The mixture was extracted thrice with diethyl ether (6 mL), and the extracts were combined, dried (anhydrous sodium sulfate), and evaporated; yield, 0.84 g (84%); specific activity, 0.59 Ci.mol^{-1}. The radioactive compound co-migrated with authentic 4-penten-1-ol

(R_F 0.46) in t.l.c. with 3:1 EtOAc–petroleum ether (b.p. 30–60), and no trace of 4-pentenal (R_F 0.81) could be detected.

[1-^{3}H]-4-Pentenyl 2,3,6-tri-O-acetyl-4-O-(tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (**5**). — Dry [1-^{3}H]-4-penten-1-ol (**3**; 0.46 g, 5.3 mmol), 1,1,3,3-tetramethylurea (0.92 g, 7.9 mmol) and hepta-*O*-acetylcellobiosyl bromide[16] (**4**; 2.55 g, 3.65 mmol) in dichloromethane (15 mL) were stirred with the exclusion of water, under nitrogen for 30 min and then cooled to −40°. Silver triflate (1.11 g, 4.33 mmol) was added during 15 min. After 30 min at −40°, the mixture was allowed to warm to room temperature, kept overnight with gentle stirring, filtered through Celite 545, and the solids were washed with dichloromethane. The filtrates were combined, successively washed with saturated sodium hydrogencarbonate and water, dried (anhydrous sodium sulfate), and evaporated under diminished pressure. Crystals of **5** were obtained from 95% ethanol; yield, 0.81 g (32%); m.p. 162–163° (lit.[5] m.p. 161°); ^{13}C-n.m.r. data (125 MHz): δ 170.46–169.03 (7 COCH$_3$), 137.77 (CH$_2$CH=CH$_2$), 115.05 (CH$_2$$C$H=CH$_2$), 100.75, 100.66 (C-1,1′), 76.51 (C-4), 72.94, 72.65, 72.56, 71.96, 71.64, 71.64 (C-2,3,5,2′,3′,5′), 69.29 (OCH$_2$–CH$_2$–), 67.86 (C-4′), 61.92, 61.59 (C-6,6′), 29.60 (OCH$_2$$CH_2CH_2$), 28.57 (OCH$_2CH_2$$CH_2$–), and 20.81–20.50 (7 COCH$_3$).

[1-^{3}H]-4,5-Epoxypentyl 2,3,6-tri-O-acetyl-4-O-(tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (**6**). — A solution of **5** (0.80 g, 1.14 mmol) in dichloromethane (7 mL) was stirred, and 3-chloroperoxybenzoic acid (0.61 g, 3.0 mmol) was added during 10 min. The reaction was monitored by t.l.c. using 1:1 toluene–ethyl acetate, and was complete after 3 h at 25°. The mixture was cooled to 0° and filtered, and the filtrate was washed twice with saturated sodium hydrogencarbonate and water, dried (anhydrous sodium sulfate), and evaporated under diminished pressure; crystallization of the resulting syrup from 95% ethanol gave epoxide **6**; yield, 0.55 g (67%); m.p. 154–155° (lit.[5] m.p. 154°); ^{13}C-n.m.r. data (125 MHz): δ 170.44–168.98 (7 COCH$_3$), 100.84, 100.66 (C-1,1′), 76.44 (C-4), 72.90, 72.62, 72.47, 71.90, 71.53, 71.49 (C-2,3,5,2′,3′,5′), 69.46 (OCH$_2$CH$_2$–), 67.77 (C-4′), 61.86, 61.52 (C-6,6′), 51.81 ($-CH_2\overbrace{\mathit{C}H-CH_2}^{O}$), 46.93 ($-CH_2\overbrace{CH-\mathit{C}H_2}^{O}$), 27.97, 27.64 (OCH$_2$$CH_2CH_2$–, two isomers), 25.79, 25.42 (OCH$_2$CH$_2$$CH_2$–, two isomers); and 20.80–20.47 (7 COCH$_3$).

[1-^{3}H]-4,5-Epoxypentyl 4-O-β-D-glucopyranosyl-β-D-glucopyranoside (**7**). — *O*-Deacetylation of the *O*-acetylated epoxypentyl cellobioside **6** was performed according to the procedure of Herzig and Nudelman[9]. The hepta-*O*-acetyl cellobioside **6** (0.45 g, 0.63 mmol) was suspended in a slurry of MgO (0.4 g) in methanol (10 mL) and incubated at 45° with stirring. The reaction was monitored by t.l.c. using 17:2:1 EtOAc–MeOH–H_2O, and, after 48 h, all traces of acetylated intermediates had disappeared. The suspension was filtered, and the filtrate was evaporated to dryness *in vacuo;* yield, 0.182 g (68%); specific activity, 0.36 Ci.mol^{-1}; ^{13}C-n.m.r. data (125 MHz): δ 130.29, 102.74 (C-1,1′), 79.49 (C-4), 76.69, 76.24,

75.44, 75.06 (C-3,5,3′,5′), 73.87, 73.61 (C-2,2′), 70.58 (OCH_2CH_2–), 70.19 (C-4′), 61.32, 60.82 (C-6,6′), 54.46 (), 48.94 (), 28.85 ($-OCH_2CH_2CH_2-$), and 26.05 ($-OCH_2CH_2CH_2-$).

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