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EPOXYALKYL OLIGO-(1→4)-β-D-GLUCOSIDES AS ACTIVE-SITE-DIRECTED INHIBITORS OF CELLULASES

GÜNTER LEGLER* AND ERNST BAUSE

Organisch-Chemisches Institut der Universität, Bonn (Deutschland)

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ABSTRACT

Eight alkyl oligo-β-D-glucosides having a terminal epoxide function have been synthesized and tested as active-site-directed, irreversible inhibitors for cellulases from an *Oxyporus* species, *Aspergillus niger*, and *Asp. wentii*. The D-glucosides showed very little reactivity, but increasing deactivation was observed with an increased number of D-glucose residues. Variation of chain length of the aglycon group showed a maximum of reactivity with 5 carbon atoms. This and the partial protection of the enzyme by cellobiose indicate that the deactivation is due to attack at the active site. The variation of the deactivation rate with pH is very similar to that of the cellulase activity, and, in each case, a carboxyl group seems to be involved as essential acid-catalyst. In the *Oxyporus* preparation, a cellobiosidase activity was detected with *p*-nitrophenyl β-cellobioside as substrate. This activity is due to a separate enzyme because it was not affected by (1,3/2,4)-5-cyclohexene-1,2,3,4-tetrol epoxide, which is a specific inhibitor of β-D-glucosidase, and showed only little reactivity with one of the cellulase inhibitors.

INTRODUCTION

Cellulases (β-1,4-glucan 4-glucanohydrolases, E.C. 3.2.1.4) hydrolyse β-(1→4)-D-glucosidic linkages in cellulose. In addition, they can hydrolyse oligosaccharides with at least four β-(1→4)-linked D-glucose residues[1]. For an investigation of their mechanism of action, it is desirable to have an active-site-directed, irreversible inhibitor that would permit labelling of the functional groups involved in the catalytic process. Preliminary experiments showed that conduritol-B [(1,3/2,4)-5-cyclohexene-1,2,3,4-tetrol] epoxide, which is specific for β-D-glucosidases[2], is without effect on cellulase activity. Oligosaccharides containing an epoxide function were therefore synthesised and their interaction with the enzyme was investigated.

The compounds listed in Table I were prepared by reaction of the acetylated α-D-glycosyl bromides with an unsaturated alcohol, oxidation of the double bond with *p*-nitroperoxybenzoic acid, and subsequent deacetylation with sodium methoxide.

*Present address: Institut für Biochemie der Universität Köln, D-5 Köln 1, Zülpicher Str. 47, Deutschland.

The deactivation experiments were carried out with commercial cellulases from *Oxyporus* sp., *Aspergillus wentii*, and *Asp. niger*. The β-D-glucosidases present in these preparations were deactivated by incubation in 10mM conduritol-B epoxide to prevent the destruction of the cellulase inhibitors. The enzymes were then incubated with the inhibitors, and the degree of deactivation was determined by viscometric determination of cellulase activity. The behaviour of a β-cellobiosidase present in the *Oxyporus* preparation against some of the inhibitors was investigated with *p*-nitrophenyl β-cellobioside as substrate.

EXPERIMENTAL

Materials. — The cellulases used were preparations from *Oxyporus* sp. (E. Merck, Darmstadt), 90 mU/mg, *Aspergillus niger* (Koch–Light Laboratories, Colnbrook, Bucks.), 1000 U/g, and *Aspergillus wentii* (Röhm, Darmstadt), crude extract. Carboxymethylcellulose, sodium salt, was obtained from Serva (Heidelberg). Conduritol-B epoxide was prepared according to Legler[2], *p*-nitrophenyl β-D-glucopyranoside according to Glaser and Wulwek[3], and *p*-nitrophenyl β-cellobioside according to Babers and Goebel[4]. Cellobiose octa-acetate was prepared by acetolysis of cotton wool, and cellotriose by chromatography[5] of cello-oligosaccharides on Sephadex G 25 (Pharmacia, Uppsala). 3-Buten-1-ol was prepared by reduction[6] of vinylacetic acid, and 4-penten-1-ol from tetrahydrofurfuryl alcohol[7]. 4-Methylenecyclohexanol was prepared from 4-benzoyloxycyclohexanone[8] by reaction[9] with methylenetriphenylphosphorane. The benzoate of 4-methylenecyclohexanol had b.p. 100°/0.5 mmHg (Found: C, 77.6; H, 7.6. $C_{14}H_{16}O_2$ (221.4) calc.: C, 77.75; H, 7.46%). The free alcohol had b.p. 75–77°/10 mmHg (Found: C, 75.1; H, 10.9. $C_7H_{12}O$ (112.2) calc.: C, 74.95; H, 10.78%). All compounds had n.m.r. and i.r. spectra consistent with the proposed structures.

p-Nitroperoxybenzoic acid was prepared according to Vilkas[10]. Thin-layer chromatography (t.l.c.) of the acetylated glycosides was performed on Silica Gel G (Merck) in benzene–acetone–methanol (90:6:3). The spots were revealed by spraying with 25% chlorosulphonic acid in acetic acid and heating to 120°.

Synthesis of glycosides. — The acetylated glycosyl bromides of D-glucose, cellobiose, and cellotriose were prepared essentially by the method of Körösy and Bárczai-Martos[11]. With cellobiose and cellotriose, it was necessary to first dissolve the acetates in chloroform and then carry out the bromination.

To a solution of the α-D-glycosyl bromide acetate (1.5 g) in dry chloroform (30 ml), the appropriate alcohol (30 ml), calcium sulphate hemihydrate (10 g), silver carbonate (2 g), and a crystal of iodine were added. The mixture was stirred for 12 h in the dark, filtered with the aid of Celite, and concentrated to dryness. The residue was crystallized from ethanol. The recrystallized cellobioside hepta-acetates were always contaminated (t.l.c.) with cellobiose hepta-acetate. Based on the epoxide content of the epoxide hepta-acetates, this contamination was 10–20%. Because the contaminant could not be removed by further crystallisations, the 4-pentenyl,

5-hexenyl, and 4-methylenecyclohexyl cellobioside hepta-acetates were purified by preparative t.l.c. Yields and analytical and physical data are listed in Table I.

TABLE I

DATA FOR D-GLUCOSIDES AND CELLOBIOSIDES

Compound	*M.p. of acetate (degrees)*	$[\alpha]_D^{20}$ *(degrees)*[a]	*Formula*	*Calc. (%)*		*Found (%)*	
				C	*H*	*C*	*H*
D-Glucosides							
Allyl[b]	85	−25.5					
3-Butenyl	70	−23.2	$C_{18}H_{26}O_{10}$	53.72	6.51	53.1	6.42
4-Pentenyl	42	−22.5	$C_{19}H_{28}O_{10}$	54.80	6.78	54.3	6.81
Cellobiosides							
Allyl	179	−21.0	$C_{29}H_{40}O_{18}$	51.48	5.97	50.6	6.11
3-Butenyl	182	−19.5	$C_{30}H_{42}O_{18}$	52.04	6.08	51.8	5.98
4-Pentenyl	161	−23.3	$C_{31}H_{44}O_{18}$	52.83	6.30	53.1	6.52
5-Hexenyl	154	−21.0	$C_{32}H_{46}O_{18}$	53.47	6.47	53.2	6.28
4-Methylene-cyclohexyl	199	−24.8	$C_{33}H_{46}O_{18}$	54.24	6.35	53.6	6.01

[a]For 1% solutions in chloroform. [b]E. Fischer [*Z. Physiol. Chem.*, 108 (1920) 5] gave m.p. 88–90°, $[\alpha]_D$ −26.21°.

4-Pentenyl β-cellotrioside deca-acetate was purified by preparative t.l.c., with two solvent developments. The product, which formed a glassy mass with m.p. 60–70°, could not be crystallised.

Epoxidation was effected by dissolving 1 mmole of the glycoside acetate in chloroform (10 ml) and stirring with about 2 mmoles of *p*-nitroperoxybenzoic acid. The permanganate test for double bonds (a drop of the reaction mixture spotted on to filter paper and sprayed with 0.5% $KMnO_4$ in 5% Na_2CO_3) was negative after 6–10 h. The cooled (0°) and filtered reaction mixture was washed twice with 0.5M potassium hydrogen carbonate, dried ($MgSO_4$), and concentrated, and the residue was crystallized from ethanol–ether. The yields were above 80%, and the other relevant data are given in Table II.

For deacetylation, the acetates were dissolved or suspended in anhydrous methanol (10% w/v) and made 50mM with respect to sodium methoxide by the addition of a M solution. After stirring for 30 min at room temperature, the solution was neutralized with an equivalent amount of acetic acid and concentrated to a glassy foam. Attempts to crystallize the free glycosides failed, except with the compounds noted in Table II.

The epoxide content of the non-crystalline glycosides, determined by Kerkow's method[12], was 60–90%. The concentration of the solutions for the deactivation experiments was based on the epoxide content.

Determination of cellulase activity. — The substrate was a 0.5% solution of carboxymethylcellulose in 50mM sodium acetate–hydrochloric acid (pH 5.0) that

TABLE II

DATA FOR EPOXIDES

	Formula	*Calc.* (%)		*Found* (%)		*M.p.* (*degrees*)	$[\alpha]_D^{20}$ (*degrees*)[a]
		C	*H*	*C*	*H*		
D-Glucoside acetates							
Allyl	$C_{17}H_{24}O_{11}$	50.50	5.98	50.2	5.69	106	−40.1
3-Butenyl	$C_{18}H_{26}O_{11}$	51.65	6.28	51.8	5.98	65	
4-Pentenyl	$C_{19}H_{28}O_{11}$	52.77	6.53	52.5	6.21	55	
Cellobioside acetates							
Allyl	$C_{29}H_{40}O_{19}$	50.29	5.82	49.8	5.58	170	
3-Butenyl	$C_{30}H_{42}O_{19}$	50.99	5.99	51.4	6.25	178	
4-Pentenyl	$C_{31}H_{44}O_{19}$	51.66	6.15	51.2	6.02	154	
5-Hexenyl	$C_{32}H_{46}O_{19}$	52.31	6.31	51.8	6.11	145	
4-Methylene-cyclohexyl	$C_{33}H_{46}O_{19}$	53.08	6.21	52.7	5.93	156[b]	
Cellobiosides							
4-Pentenyl	$C_{17}H_{30}O_{12}$	47.88	7.09	47.5	6.92	153	−26.8
5-Hexenyl	$C_{18}H_{32}O_{12}$	49.08	7.32	48.6	7.17	149	−25.1

[a]For 0.5% solutions in water. [b]A new crystalline form appeared above 115°.

had been clarified by centrifugation at 15,000 *g* for 1 h. Viscosities were determined in an Ostwald-type viscometer (capillary diameter, 0.5 mm; efflux time of buffer, 28 sec; efflux time of substrate, 200 sec) at 25°. Since only relative values of enzyme activity were needed, the time course of viscosity decrease was not investigated, but the efflux time after a constant time of incubation (10 min at 25° with different amounts of enzyme) was measured. A standard curve was constructed by plotting the efflux time against the amount of enzyme taken.

Deactivation experiments. — Cellulase (5 mg) was dissolved in 50mM acetate buffer (1 ml, pH 5.0) and incubated with conduritol-B epoxide (2–3 mg) for 3–4 h at room temperature. No β-D-glucosidase activity could be detected with *p*-nitrophenyl β-D-glucoside after this treatment. Aliquots (0.5 ml) of this enzyme solution were pipetted on to weighed amounts of epoxyalkyl glycoside to give the desired inhibitor concentration (usually 0.1M). Samples (30 μl) were withdrawn directly after mixing, and after 1, 2, 4, and 6 h at 25°, and injected into the substrate solution (10 ml) for activity determination. Logarithms of the relative activities were plotted against time and from these first-order rate constants for the deactivation were calculated.

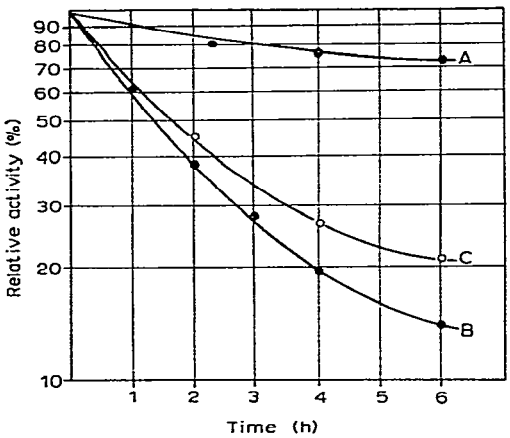
The following buffers were used to determine the influence of pH on the enzymic activity and deactivation rate: pH 4.0 to 6.0, 50mM sodium acetate–hydrochloric acid; pH 7.0 and 8.0, 50mM phosphate. With the deactivation experiments, enzyme and inhibitor were incubated in the appropriate buffer and the activity was determined at pH 5.0. In control experiments, no loss of enzyme activity or epoxide content was detected.

Determination of cellobiosidase activity. — A solution of enzyme (0.25 mg) in 50mM acetate buffer (50 μl, pH 5.0) was pipetted into 20mM *p*-nitrophenyl cello-

bioside (2 ml) in the same buffer and incubated at 25°. Samples (0.1 ml) were withdrawn every 10 min and pipetted into 5% aqueous sodium carbonate (10 ml), and the absorbance was measured at 410 nm. From this, the rate of *p*-nitrophenol release was calculated.

RESULTS AND DISCUSSION

With all three cellulase preparations, very similar results were obtained. The epoxyalkyl glucosides showed only very little reactivity (10–25% deactivation after 6 h). However, the deactivation rate increased considerably with the number of D-glucose residues (Fig. 1; note that the cellotrioside is only one-fifth of the concentration of the others).



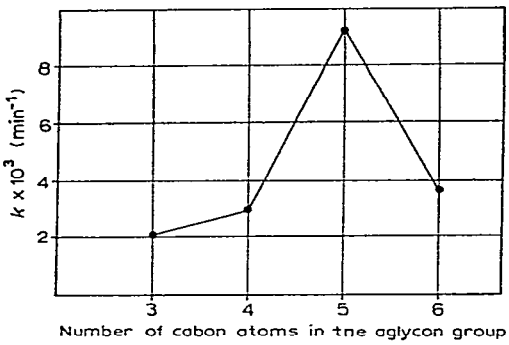
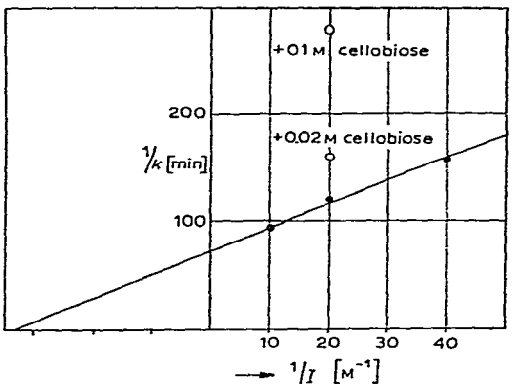


Fig. 1. Deactivation of cellulase from *Oxyporus* sp. at pH 5.0 and 25° by (*A*) 4,5-epoxypentyl β-D-glucopyranoside (50mM), (*B*) 4,5-epoxypentyl β-cellobioside (50mM), (*C*) 4,5-epoxypentyl β-cellotrioside (10mM).

Fig. 2. First-order rate constants for the deactivation of *Oxyporus* sp. cellulase by 0.1M epoxyalkyl β-cellobiosides of different chain-length at pH 5.0 and 25°; because of the competitive inhibition by cellobiose contaminant, the rates for epoxypropyl and epoxybutyl cellobioside might be 10 and 15% too low.

The observed dependence of the deactivation rate on the number of D-glucose residues and on the distance of the epoxide group from the glucosyl oxygen atom (Fig. 2) shows that the loss of cellulase activity is not due to some non-specific reaction but is the result of an attack at the active site. This is also supported by the decrease in deactivation rate in the presence of cellobiose and by its concentration dependence (Fig. 3). The linear, double-reciprocal plot points to the reversible formation of an enzyme inhibitor complex that precedes the deactivation proper.

The maximum of reactivity with five carbon atoms in the aglycon group (Fig. 2) would indicate that, with this chain length, the epoxide is able to reach a



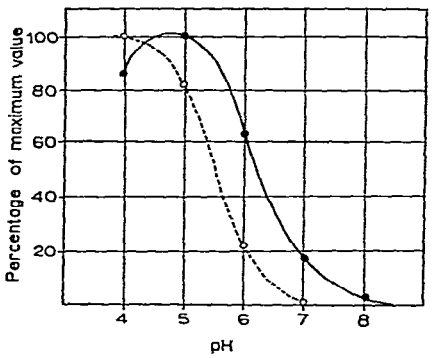
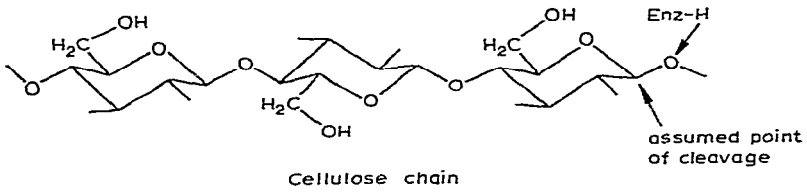
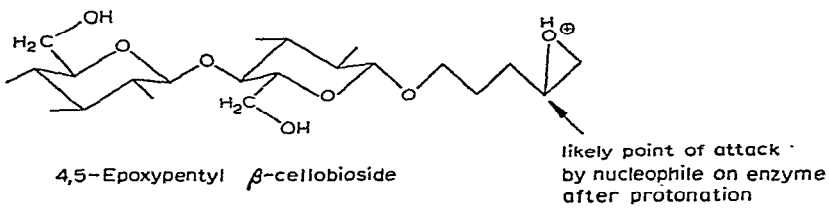


Fig. 3. Double reciprocal plot of the deactivation of cellulase from *Oxyporus* sp. by 4,5-epoxypentyl β-cellobioside at pH 5.0 and 25°. The intercept with the abscissa gives $K_I = 0.033$M.

Fig. 4. Cellulase activity (——●) of *Oxyporus* enzyme and deactivation rate with 50mM 4,5-epoxypentyl β-cellobioside (----○) as a function of pH at 25°.

position on the enzyme corresponding to the glucosyl oxygen atom of the last D-glucose residue of a cellotriose section of cellulose (Scheme 1). In accordance with Whitaker's results on the hydrolysis of oligosaccharides from cellulose[1], it may be assumed that two or three binding-sites for adjacent D-glucose residues are present at the active centre of cellulase and that the catalytic site is in a position corresponding to the glycosyl oxygen atom of the next D-glucose residue towards the reducing end.





Scheme 1. All of the epoxides investigated are probably mixtures of diastereoisomers, with approximately equal proportions of the *R* and *S* configurations at the secondary carbon atom of the epoxide group.

Compared to the Michaelis constant (2×10^{-5} M per D-glucose residue[13]) of carboxymethylcellulose, the dissociation constant (0.033M) of the enzyme inhibitor complex obtained from Fig. 3 is rather high. This could mean that an additional D-glucose binding-site (unable to interact with the inhibitor because of the hydrocarbon nature of the side chain) is present directly at the catalytic site, and/or additional binding-sites are present towards the reducing end of the cellulose chain. The latter assumption is supported by Whitaker's results on the cleavage site in higher cello-oligosides[1]. The situation would then be similar to lysozyme, where there are three binding-sites on one side of the catalytic site and three on the other[14]. An analogous, covalent inhibition of lysozyme has been published by Thomas *et al.*[15], who used 2,3-epoxypropyl β-glycosides of 2-acetamido-2-deoxy-D-glucose oligomers. Comparable deactivation rates were, however, obtained at much lower concentrations. Since a reduction in the rotational degrees of freedom in the aglycon group might increase the effectiveness of the inhibitor, provided that the distance between the glycosyl oxygen atom and the epoxide function were kept constant, 4-methylenecyclohexyl β-cellobioside was synthesised. On treatment with *p*-nitroperoxybenzoic acid, this should give an approximately equimolar mixture of the *cis* and *trans* epoxide, with perhaps a slight preponderance of the *trans* isomer. From a study of Dreiding models, we may expect the epoxide oxygen atom of the latter isomer to take up a position corresponding to that of the glycosyl oxygen atom within 0.4 Å. However, the epoxide was completely unreactive at a concentration of 80mM for the epoxide mixture. At present, we cannot give a satisfactory explanation for this.

The pH dependence of the deactivation rate and of the cellulase activity is shown in Fig. 4. Both curves point to the participation of a carboxyl group in the catalytic process and in the protonation of the epoxide. The deactivation might, therefore, proceed in a manner similar to that of the β-D-glucosidases investigated previously[16]. Another explanation might be that protonation of a carboxylate group is necessary for the maintenance of the catalytically active conformation of the enzyme.

In the *Oxyporus* preparation, a cellobiosidase activity was detected with a specific activity of 2.8 μmoles of *p*-nitrophenyl β-cellobioside.min^{-1}.mg^{-1}. This activity was not influenced by conduritol-B epoxide. The deactivation rate with 4,5-epoxypentyl β-cellobioside was ~20% of the rate of the cellulase deactivation with this inhibitor. These observations show that the cellobiosidase activity is due to a separate enzyme and that its specificity probably requires the intact cellobiose structure. Another, though less likely, explanation for the ineffectiveness of the epoxy glucoside would be that this cellobiosidase acts by a mechanism not related to that of the β-D-glucosidases and cellulases.

The deviations from purely first-order kinetics, as shown in Fig. 1, might be due to hydrolysis of the inhibitors by this cellobiosidase, but it might also be explained by the presence of different cellulases reacting at different rates or by an enzyme-catalyzed hydrolysis of the epoxide as observed with β-D-glucosidases[16]. If the deviations are due to the presence of different cellulases, the rates plotted in Figs. 2–4 would refer to the most-reactive species present.

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