ACTIVE SITE-DIRECTED INHIBITION OF GALACTOSIDASES BY CONDURITOL C EPOXIDES (1,2-ANHYDRO-*EPI*- AND *NEO*-INOSITOL)

Günter LEGLER and Monika HERRCHEN
Institut für Biochemie der Universität zu Köln, Zülpicher Str. 47, 5000 Köln 1, FRG

Received 11 September 1981

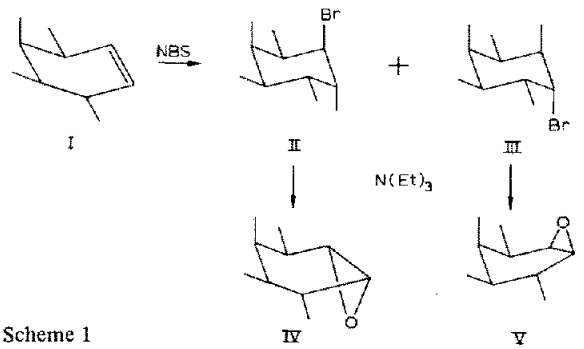
1. Introduction

1,2-Anhydroinositols with a configuration of their hydroxyl groups corresponding to that of the substrate glycose residue are potential active site-directed inhibitors for glycohydrolases for 2 reasons:

(i) They can be bound specifically by interaction with complementary groups of the substrate binding site;

(ii) The epoxide function can be activated by a suitably oriented acidic group, subsequently forming a covalent bond with a nucleophilic group at the catalytic site [1].

While this principle has been applied successfully to the labeling of β- and α-glucosidases [2–4] there is only an unpublished exploratory study for the analogous reaction with β-galactosidases [5]. The difficulties encountered during the synthesis of pure 1,2-anhydro-*epi*-inositol (V) based on the reactions depicted in scheme 1 have now been overcome by a new method for the preparation of conduritol C (I) [6] and by the application of partition chromatography on ion exchange resins [7] for the separation of the bromohydrins II and III.



Scheme 1

Abbreviations: NBS, *N*-bromosuccinimide; 4-MeUmb, 4-methyl-umbelliferyl-

Here, we report the results of inhibition studies with the epoxides V and IV and β- and α-galactosidases from 5 different sources.

2. Materials and methods

2.1. *Conduritol C* cis *and* trans-*epoxide (V and IV, respectively)*

Conduritol C (300 mg) synthesized according to [6], was dissolved in 15 ml 0.4% aqueous acetic acid and stirred with 600 mg NBS for 16 h at room temperature protected from light. Excess NBS was destroyed by the addition of 100 mg $NaHSO_3$ and the solution taken to dryness by vacuum evaporation. The residue was taken up in 50 ml ethanol, filtered from inorganic salts and concentrated to a syrup.

To facilitate the control of subsequent chromatographic separations of the bromination products [^{3}H]conduritol C was prepared with $NaBH^3H_4$ and treated with NBS as above to give a mixture of 4 mg (2×10^7 cpm) [^{3}H]bromohydrins. A part of this was applied to a column (2 × 50 cm) with Dowex 50W × 4, 200–400 mesh, Li^+-form equilibrated with 2-propanol/water, 9:1 (v/v). Elution with the same solvent gave 3 peaks at 135–180 ml (1), 190–230 ml (2) and 300–360 ml (3) with relative magnitude 1:1.4:1.5. Peaks (2) and (3) consisted of bromohydrin II and III, respectively.

A preparative scale separation with the bromohydrin mixture from 300 mg conduritol C was done on the same column. II and III were crystallized from ethanol/ether.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

2.2. *(1,2,3,6/4,5)-6-bromo cyclohexanepentol (II), m.p. 155°C*

(1,2,3,4/5,6)-6-bromo cyclohexanepentol (III), m.p. 162°C

II and III were converted to the respective epoxides by dissolving 100 mg in 10 ml water and maintaining the pH at 10.5–11 by intermittent additions of 0.1 N triethylamine. Consumption of base ceased after 1 h. The solutions were concentrated to a small volume to remove excess triethylamine, diluted with water to 10 ml and filtered at 5°C over 10 g Dowex 1 × 4, OH^--form. The effluent was neutralized by the addition of Dowex 50, H^+-form, taken to dryness and the residue crystallized from ethanol/ether.

2.3. *Conduritol C* trans-*epoxide (IV, from peak (2)) m.p. 145°C*

The material is identical (TLC, m.p.) to the epoxide obtained by the oxidation of conduritol C with peroxybenzoic acid [8].

2.4. *Conduritol C* cis-*epoxide (V, from peak (3)), m.p. 126°C*

Control of purity by TLC on silical gel was done in ethanol/ethyl acetate/water 2:8:1 (by vol.). R_F-values are: II 0.65, III 0.55, IV 0.43 and V 0.38.

2 mg each of IV and V in 0.2 ml water were hydrolyzed for 12 h at 100°C in the presence of 50 mg Dowex 50, H^+-form and subjected to paper chromatography in pyridine/ethyl acetate/water, 1:3.6:1.2 (by vol.), upper phase (solvent 1) and in acetone/water, 5:1 (v/v) (solvent 2) with *chiro*-, *epi*- and *myo*-inositol as reference. The following relative R-values were observed: solvent 1, *chiro*-1.00, *epi*-0.80, *myo*-0.07, inositols from IV and V 1.9 and 0.65; solvent 2, *chiro*-1.00, *epi*-0.85, *myo*-0.44, inositols from IV and V 1.17 and 0.77. Both IV and V gave the same 2 spots with the same relative intensities, the slower migrating substance amounting to ~5% of the faster one.

Reaction rates of IV and V with thiosulfate were measured photometrically at 530 nm by adding 20 μmol epoxide to 2.0 ml 0.1 M $Na_2S_2O_3$ in 10% aqueous ethanol containing 0.025% phenolphthalein.

2.5. *Substrates and reversible inhibitors*

4-MeUmb-β-D-galactoside and -glucoside were synthesized according to [9], 4-MeUmb-α-D-galactoside and methyl-β-D-1-thiogalactoside were from Sigma (München), D-galactal was prepared according to [10]. Other chemicals were of reagent grade.

2.6. *Enzymes*

β-Galactosidase from *Escherichia coli* was purchased from Boehringer (Mannheim) [1] and from Sigma (München) [2]. Another sample [3] was provided by Dr A. Fowler (Los Angeles CA). The following kinetic parameters were found with 4-MeUmb-β-galactoside at pH 7.0 and 25°C in the presence of 1 mM Mg^{2+} (values in brackets with 10 mM EDTA instead of Mg^{2+}):
1. V_{max} 120 (2.0) U/mg protein; K_m 0.14 (1.04) mM
2. V_{max} 270 (4.2) U/mg protein; K_m 0.14 (1.00) mM
3. V_{max} 86 (1.6) U/mg protein; K_m 0.17 (1.00) mM

β-Galactosidase from *Aspergillus wentii* was prepared from a spray-dried culture filtrate from Röhm GmbH (Darmstadt) by ammonium sulfate fractionation and chromatography on CM- and DEAE-Sephadex [11]. It is characterized by the following properties: M_r 160 000, isoelectric point 4.7, E_{280} 1.80 ml . mg^{-1} . cm^{-1}; activity with 4-MeUmb-β-galactoside at pH 4.0 and 25°C, V_{max} 106 U/mg, K_m 0.73 mM; activity with 1 mM 4-MeUmb-β-glucoside <0.5 U/mg, K_i for D-galactal 13 μM (no lag in the approach to the steady state).

β-Glucosidase A from sweet almonds was isolated from a crude β-glucosidase preparation from Sigma (München) as in [12]. β-Glucosidase and β-galactosidase activities at pH 5.0 were 222 and 8.7 U/mg protein, respectively.

A crude β-glucuronidase preparation from *Helix pomatia* (type H-II from Sigma, München) was used as source of β-glucosidase/β-galactosidase from this organism. β-Glucosidase and β-galactosidase activities at pH 5.0 were 4.5 and 1.87 U/ml, respectively.

β-Galactosidase and β-glucosidase was from calf spleen: fresh tissue was homogenized at 4°C with 2.5 vol. 10 mM phosphate (pH 6.0) containing 0.3% Triton X-100 and centrifuged at 3000 × g for 30 min. The supernatant was diluted with 10 vol. 100 mM sodium citrate (pH 4.6) and left at 4° for 16 h. The precipitate collected by 30 min centrifugation at 10 000 × g was used as enzyme source after resuspension in 10 mM phosphate (pH 6.0) containing 1% Triton X-100. β-Galactosidase and β-glucosidase activites were 10 000 and 14 000 nmol . h^{-1}. 10 g $tissue^{-1}$, respectively.

α-Galactosidase from coffee beans was from Sigma (München).

2.7. *Activity measurements and inhibition studies*

Glycosidase activities except for the calf spleen enzymes were determined fluorimetrically with the respective 4-MeUmb-glycosides as in [13]. Substrate concentrations were 0.5 mM galactoside and 1 mM glucoside except for determinations of V_{max}, K_m and K_i. Buffers (all 50 mM) were as follows: pH 2.5–3.5, glycine/HCl; pH 4.0–5.5, sodium acetate/HCl; pH 6.0–8.5, sodium phosphate/HCl. Buffer solutions for β-galactosidase from *E. coli* contained, in addition, 145 mM NaCl and 1 mM Mg^{2+} or 10 mM EDTA.

Activities of the calf spleen enzymes were determined at pH 5.0 and 37°C as in [14] except that substrate solutions contained 0.3% Triton X-100 and 1% sodium taurocholate.

Inhibition was measured by incubating the enzyme with appropriate concentrations of epoxide at 25°C and taking samples at various time intervals for activity measurement. Reversibility of the inhibition was tested by activity determination after gel chromatography on Sephadex G-25 or continuous flow dialysis (18 h) in a 1 ml flow cell with 600 ml buffer. Complete removal of unbound inhibitor was checked by the disappearance of [^{14}C]glucose added prior to gel chromatography or dialysis.

3. Results and discussion

3.1. *Conduritol C* cis- *and* trans-*epoxide (1,2-anhydro-* epi- *and* neo-*inositol)*

Our structure assignment for the two expoxides is based on the following findings: IV is identical to the epoxide obtained by direct epoxidation of conduritol C with peroxybenzoic acid [8]. Steric hindrance by the axial hydroxyl group is expected to direct the peroxy acid to the *trans*-position. Since both IV and V give the same inositols on acid hydrolysis their only difference must be the orientation of the epoxide ring which is, therefore, *cis* to the axial hydroxyl group in V. This is supported by the different reaction rates with thiosulfate which is 1.3-times faster with the less hindered V.

3.2. *Inhibition of galacto- and glucosidases*

The reactivity of the epoxides with respect to their inhibition of glycohydrolases may be characterized as follows (see table 1 for kinetic data).

Specific β-galactosidases, e.g., the enzymes from *E. coli* [16] and from *Asp. wentii* [11] and β-glycosidases that catalyze the hydrolysis of both galacto- and glucosides, e.g., from *Helix pomatia* [17] and the β-glucosidases from almonds [18] are readily inactivated by the *cis*-epoxide V and not by the *trans*-isomer IV. Only the latter enzymes are also inactivated by the glucosidase-specific conduritol B epoxide [5].

β-Glucosidases which are strictly specific for glucosides are not inhibited by V (e.g., β-glucosidases A_3 from *Asp. wentii* [19], not shown) or their reaction with V is several orders of magnitude slower than with conduritol B epoxide with its configuration corresponding to that of glucose (e.g., β-glucosidase from calf spleen, table 1).

α-Galactosidase from coffee beans is only inhibited by IV and not by V.

Table 1
Kinetic parameters for the time-dependent inhibition of galacto- and glucosidases by conduritol C epoxide

Enzyme	k_i(max)[a] (min^{-1})	K_m[a] (mM)	k_i(max)/K_m ($min^{-1} \cdot M^{-1}$)
β-Galactosidase			
E. coli, pH 7.0	0.052	0.23	230
E. coli, Mg^{2+}-free	0.001	0.16[b]	–
β-Galactosidase			
Asp. wentii, pH 4.0	1.4	1.0	1400
β-Gluco-/galactosidase			
Helix pomatia, pH 5.0			
β-glucosidase activity	n.d.	n.d.	8.2[c]
β-galactosidase activity	n.d.	n.d.	11[c]
β-Glucosidase A			
sweet almonds, pH 5.0			
β-glucosidase activity	n.d.	n.d.	11.8[c]
β-galactosidase activity	n.d.	n.d.	12.0[c]
β-Galactosidase			
calf spleen, pH 5.0	n.d.	n.d.	10[d]
β-Glucosidase			
calf spleen, pH 5.0	n.d.	n.d.	3.4[d]
calf spleen[e]	n.d.	n.d.	5000[e]
α-Galactosidase [f]			
coffee beans, pH 5.0	n.d.	n.d.	6.6[f]

[a] Constants calculated from the first-order rate constants k_i for the activity loss with various inhibitor concentrations [I] according to: $k_i = k_i(max)\,[I]/K_m + [I]$

[b] Inhibition constant for the competitive inhibition of 4-MeUmb-β-galactoside hydrolysis

[c] Figures represent $k_i/[I]$ with 1 mM epoxide

[d] As for [c] with 5 mM epoxide

[e] As for [c] with 0.01 mM conduritol B epoxide

[f] No inhibition was observed with *cis*-epoxide, $k_i/[I]$ is calculated from activity loss with 5 mM conduritol C *trans*-epoxide

Detailed kinetic studies were made with the β-galactosidases from *E. coli* and from *Asp. wentii*. The first-order rate constant for the time-dependent inhibition did not linearly depend on the inhibitor concentration but could be described by a rate law corresponding to the Michaelis-Menten equation. The presumably covalent reaction with the enzyme is thus preceded by the formation of a non-covalent complex with a dissociation constant K_m (table 1) comparable to K_i for β-galactose.

pH-Dependence of the inhibition (fig.1) closely parallels that for k_{cat} for *O*-galactoside hydrolysis by the 2 enzymes. That the activity loss is due to a reaction of the epoxide at the active site is also shown by the protection of the enzymes by competitive reversible inhibitors. The decrease of the inhibition rate in the presence of such inhibitors is in good agreement with the decrease expected from theory (table 2).

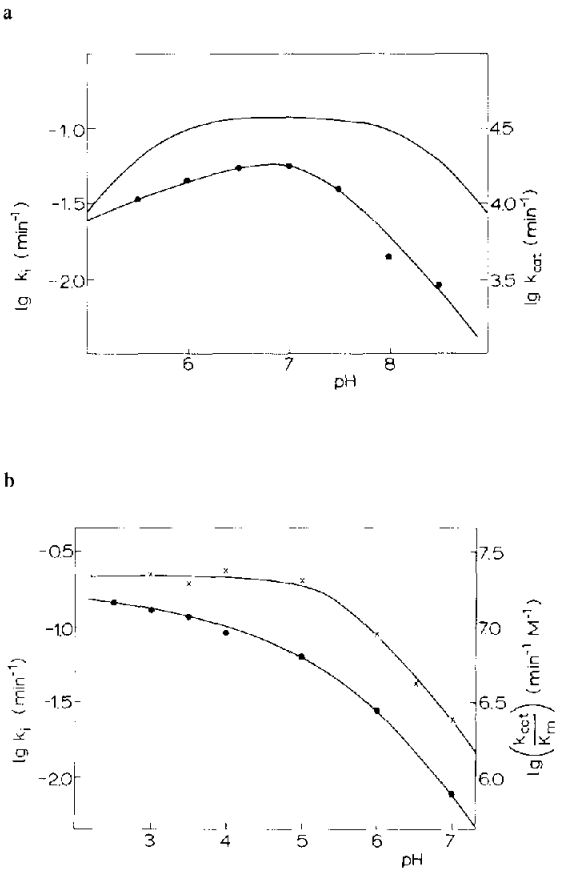


Fig.1. pH-Dependence of the inhibition rate constant k_i by conduritol C *cis*-epoxide of β-galactosidases from *E. coli* (a) with 5 mM epoxide and from *Asp. wentii* (b) with 0.05 mM expoxide (left ordinate, lower curve). pH-Dependence of k_{cat} for the hydrolysis of 2-nitrophenyl-β-galactoside [25] (a) and V_{max}/K_m for 4-MeUmb-β-galactoside [11] (b) (right ordinate, upper curve) is included for comparison. The latter parameter was chosen for the *Asp. wentii* enzyme because inhibition rates were measured with $[I] \ll K_m$.

Table 2
Inhibition of the reaction of conduritol *cis*-epoxide with β-galactosidases by competitive reversible inhibitors

Enzyme source	Inhibitor	$C_{epoxide}$ (mM)	$\frac{k_i(O)}{k_i(I)}$ calc.	$\frac{k_i(O)}{k_i(I)}$ found
E. coli	Methyl-β-D-1-thiogalactoside	0.1	2.35	2.23
	3.4 mM	0.3	1.82	2.00
Asp. wentii	D-Galactal 0.027 mM	0.5	2.33	2.6

[a] The ratio of first-order rate constants for the activity loss due to the reaction of conduritol C *cis*-epoxide in the absence ($k_i(O)$) and in the presence ($k_i(I)$) of the competitive reversible inhibitor is calculated from:

$$\frac{k_i(O)}{k_i(I)} = 1 + \frac{C_{comp}/K_i}{1 + (C_{epox}/K_m)}$$

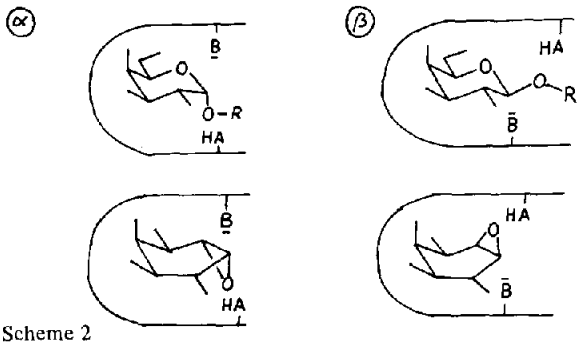
K_i is 1.7 mM for methyl-β-D-1-thiogalactoside [20] and 0.013 mM for D-galactal [11]. K_m was taken from table 1. C_{comp}, concentration of competitive reversible inhibitor

Activity loss with time was a first-order process down to ~1% residual activity. At higher degrees of inhibition the reaction slowed down and at the same time we observed a slow reactivation (e.g., from 0.05–0.3% residual activity) during the activity determinations. This reactivation occurred to the same extent with all 3 samples of the *E. coli* enzyme and it also started from the same low residual activity after gel chromatography or dialysis of the inhibited enzyme. No satisfactory explanation for this phenomenon can be given at present.

β-Galactosidase from *E. coli* requires Mg^{2+} for full activity; binding of 1 Mg^{2+}/subunit is necessary for acid catalysis in the hydrolysis of *O*-galactosides: substrate bond cleavage is 50-times slower in its absence [21]. This necessity for acid catalysis appears to be required to at least the same extent for the covalent inhibition by V: with the Mg^{2+}-free enzyme we observe only reversible inhibition.

With β-glucosidase A from almonds β-galacto- and β-glucosidase activity decrease at the same rate. This constitutes a definite proof that hydrolysis of both types of substrate is catalyzed by the same active site discussed [22,23]. A similar lack of specificity is observed with the enzyme(s) from *Helix pomatia.*

The inertness of the α-galactosidase against the *cis*-epoxide compared to its reactivity with the *trans*-isomer points to similar stereochemical relations between α- and β-specific enzymes as was observed with α- and β-glucosidases [24]. It was deduced from the conversion of conduritol B epoxide by these enzymes to *scyllo*- and (+)-*chiro*-inositol, respectively, that proton-donating and nucleophilic groups at the active site are in inverted positions with respect to their substrates. These considerations can now be extended to α- and β-galactosidases, resulting in the relations shown in scheme 2. If this is correct, derivatives of *myo*- and *neo*-inositol should be formed by α- and β-galactosidases, respectively.



Scheme 2

α-Galactosidase β-Galactosidase

4. Conclusion

These data show that the principle of active site-directed inhibition by epoxides of appropriate structure can be extended to galactosidases with the same generality as shown for glucosidases [1], thus pointing to great similarities in mechanism.

Acknowledgements

We thank Dipl.-Biol. G. Coenen and U. Petzold for the isolation of β-galactosidase from *Asp. wentii* and β-glucosidase A from almonds, respectively. Our thanks are also due to Dr A. Fowler for a sample of β-galactosidase from *E. coli*, to Dr H. Uhlig, Röhm GmbH, for the spray-dried culture filtrate from *Asp. wentii*, and to the 'Fonds der Chemischen Industrie' and the 'Deutsche Forschungsgemeinschaft' for financial support.

References

[1] Legler, G. (1977) Methods Enzymol. 46, 368–381.
[2] Bause, E. and Legler, G. (1974) Z. Physiol. Chem. 355, 438–442.
[3] Quaroni, A. and Semenza, G. (1976) J. Biol. Chem. 251, 3250–3253.
[4] Legler, G. and Harder, A. (1978) Biochim. Biophys. Acta 524, 102–108.
[5] Legler, G. (1973) Mol. Cell. Biochem. 2, 31–38.
[6] Stegelmeier, H. (1979) Thesis, University of Cologne.
[7] Samuelson, O. and Strömberg, H. (1968) Acta Chem. Scand. 22, 1252–1258.
[8] Legler, G. (1966) Z. Physiol. Chem. 345, 197–214.
[9] Robinson, D. (1956) Biochem. J. 62, 39–44.
[10] Roth, W. and Pigman, W. (1963) Methods Carbohydr. Res. 2, 405.
[11] Coenen, G. (1980) Thesis, Diplom. Biology, Univ. Cologne.
[12] Legler, G. and Hasnain, S. N. (1970) Z. Physiol. Chem. 351, 25–31.
[13] Legler, G. (1978) Biochim. Biophys. Acta 524, 94–101.
[14] Raghavan, S. S., Rhoads, D. B. and Kanfer, J. N. (1972) Biochim. Biophys. Acta 268, 755–760.
[15] Paulsen, H., Behre, H. and Herold, C. P. (1970) Fortschr. Chem. Forsch. 14, 473–524.

[16] Wallenfels, K. and Weil, R. (1972) in: The Enzymes (Boyer, P. D. ed) 3rd edn, vol. 7, pp. 618–648, Academic Press, New York.
[17] Got, R. and Marnay, A. (1968) Eur. J. Biochem. 4, 240–246.
[18] Helferich, B. and Kleinschmidt, T. (1967) Z. Physiol. Chem. 348, 753–758.
[19] Roeser, K.-R. and Legler, G. (1981) Biochim. Biophys. Acta 657, 321–333.
[20] DeBruyne, C. K. and Yde, M. (1977) Carbohydr. Res. 56, 153–164.
[21] Sinnott, M. L., Withers, S. G. and Viratelle, O. M. (1978) Biochem. J. 175, 539–546.
[22] Grover, A. K. and Cushley, R. J. (1977) Biochim. Biophys. Acta 482, 109–124.
[23] Walker, D. E. and Axelrod, B. (1978) Arch. Biochem. Biophys. 187, 102–107.
[24] Braun, H., Legler, G., Deshusses, J. and Semenza, G. (1977) Biochim. Biophys. Acta 483, 135–140.
[25] Tenu, J.-P., Viratelle, O. M., Garnier, J. and Yon, J. (1971) Eur. J. Biochem. 20, 363–370.