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A bireactant, irreversible, active-site-directed inhibitor of β-D-galactosidase (*Escherichia coli*). Synthesis and properties of (*1/2,5,6*)-2-(3-azibutylthio)-5,6-epoxy-3-cyclohexen-1-ol*

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ABSTRACT

(*1/2,5,6*)-2-(3-Azibutylthio)-5,6-epoxy-3-cyclohexen-1-ol** (**1**) was synthesized and was found to irreversibly inactivate β-D-galactosidase (*Escherichia coli*). The inactivation was prevented by the presence of isopropyl 1-thio-β-D-galactopyranoside (IPTG). The vinyloxirane group of **1** reacted with water and other nucleophiles, especially at higher pH values. Reaction of **1** with β-D-galactosidase was slow enough so that a competitive-inhibition constant (K_i) of 29mM could be determined. The inhibition constant for (*1,2/3,6*)-6-(3-azibutylthio)-2-bromo-4-cyclohexene-1,3-diol (**2**), the precursor of the bireactant inhibitor **1**, was 13mM, while that of (*1,3/2,4*)-3-(3-azibutylthio)-5-cyclohexene-1,2,4-triol (**3**), the product formed when the reactant is allowed to react with water, was 23mM. After irradiation by light, β-D-galactosidase that had initially been treated with the bireactant compound and then digested with trypsin, showed a new pattern of elution from h.p.l.c., indicating that there was reaction at two regions of the β-D-galactosidase molecule.

INTRODUCTION

A reactant capable of reacting at two places at the active sites of glycosidases would be useful as a means of determining the geometry of the active sites and would also be important for obtaining insight into the mechanism of action of such enzymes. For such purposes, an inositol derivative having a vinyloxirane unit, resembling a monosaccharide, and connected to a chain containing a diazirine, is of potential significance. A vinyloxirane has alkylation ability at the allylic carbon atom[1] and any nucleophilic functional group is a potential reactant. Vinyloxiranes, in contrast to ordinary oxiranes[2], are highly reactive and do not need assistance by protonation of the epoxide oxygen. Irradiation of a diazirine with light generates a carbene, which is highly reactive with both nucleophiles and electrophiles.

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** All chiral cyclohexene derivatives described are racemic mixtures.

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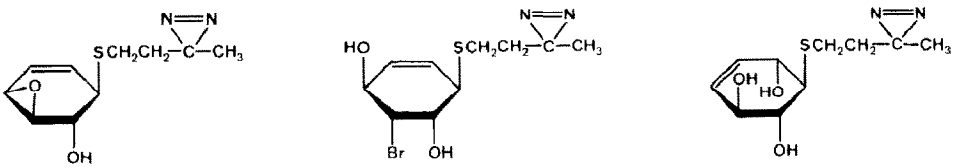
We describe here the synthesis of (*1R,2S,6*)-2-(3-azibutylthio)-5,6-epoxy-3-cyclohexen-1-ol (**1**), a unique compound having both a vinyloxirane component and a diazirine component. The compound was found to have an affinity for the active site of β-D-galactosidase and brought about an irreversible inactivation of the enzyme that was prevented by the presence of isopropyl 1-thio-β-D-galactopyranoside (IPTG), a substrate analog inhibitor of β-D-galactosidase. Irradiation by u.v. light of 350 nm did not cause any further inactivation, but some distinct changes were detected in the elution patterns of the carboxymethylated tryptic peptides when the peptides from the light-irradiated enzyme were compared to the peptides from the non-irradiated enzyme.

RESULTS AND DISCUSSION

3-Azi-1-butanethiol (**6**), generated *in situ* from its *S*-benzoate (**5**) by treatment with sodium methoxide, reacted smoothly with (*1R,4S,5R,6S*)-6-bromo-4,5-epoxy-2-cyclohexen-1-ol (**7**). The product (**2**) was subsequently converted into the respective vinyloxirane, **1**, by alkali treatment through phase-transfer catalysis. The reaction to form compound **1** was essentially quantitative, as monitoring by t.l.c. showed no formation of byproducts and the complete disappearance of the starting material **2**. Purification of **1** by column chromatography on silica gel or reversed phase chromatography using protic solvents was virtually impossible because of its chemical instability. The identity of compound **1** was, therefore, verified by showing that the expected product **3** was obtained after reaction with water and that product **8** was obtained after reaction with ethanethiol. Both products (**3** and **8**) were rigorously purified by silica gel chromatography and h.p.l.c. Because of its instability, compound **1** was employed without further purification.

Incubation of β-D-galactosidase (*E. coli*) with freshly prepared **1** caused a loss of catalyzing ability (Fig. 1). On the other hand, there was no activity loss when the enzyme was incubated with **1** in the presence of IPTG (Fig. 1). If the IPTG and other reacted components were removed after reaction (by molecular sieving) and more **1** was added, the enzyme lost activity (Fig. 1) at essentially the same rate as without the pretreatment in the presence of IPTG. In each case the loss of activity was initially first-order with respect to enzyme, but after 5–10 min the rate deviated from first-order kinetics and after 25 min the enzyme no longer lost activity. T.l.c. showed that the ability to deactivate the enzyme was lost as **1** decomposed to the hydrolysis product, **3**, showing that it was **1** that caused the inactivation and that **3** was inert. Indeed, incubation with pure compound **3** did not result in any activity loss. If more **1** was added after the initial aliquot had decomposed, another activity loss similar to the first occurred. The loss of activity as a result of incubation with **1** was irreversible, since there was no restoration of activity after a 3000-fold dilution. Incubation of this diluted enzyme in buffer also did not cause any activation as a function of time. Finally, incubation with compound **2** did not cause any irreversible loss of activity (Fig. 1). All of these reactions were conducted in the absence of light and the data indicate that the vinyloxirane caused irreversible loss of the enzymic activity.



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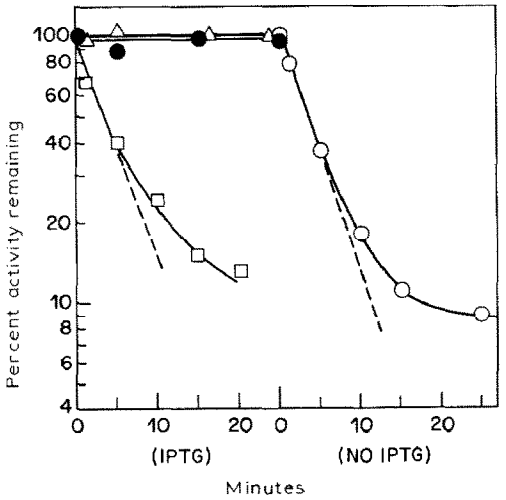


Fig. 1. Percentage of activity remaining as a function of time in assay medium that did not contain substrate. [The concentration of enzyme in each case was 1 mg/mL and the potentially reactive compounds were added at 250mM. Key: □, compound **1**; ●, compound **1** with 300mM IPTG; ○, the IPTG and other reaction components after reaction with compound **1** were removed by molecular sieving (Pharmacia LKB columns PD-10 Sephadex G-25M) and fresh compound **1** was added; △, compound **2**].

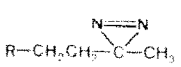
When kinetic constants with *p*-nitrophenyl β-D-galactopyranoside (PNPG) were obtained with the enzyme before addition of **1** and compared to the constants that were obtained with the activity that remained after 25 min of incubation with **1**, it was found that only the v_{max} value had changed (the v_{max} decreased from 42 to 4 U/mg). The K_m values were essentially unchanged (0.036mM before and 0.037mM after). Since the K_m value of β-D-galactosidase with PNPG as the substrate is equal to its K_s value[3], the activity loss must have been due to a change in catalytic properties, not binding effects.

Competitive inhibition constants were obtained for **1***, **2**, and **3**. The values of 29, 13, and 23mM were obtained for the three compounds, respectively. Considering the structures of the compounds, these values are acceptable, and explain why the inactivation was specific enough to be prevented by IPTG. In the case of compound **3** there was

* Very short assay times (< 1 min) were used with compound **1** so that the inhibitory activity was measured before a significant amount of enzyme inactivation or reagent decomposition had occurred.

evidence of non-competitive inhibition. The structure of **3** (as an inositol) could explain its function as a good transferolytic acceptor in the β-D-galactosidase-catalysed transfer reaction[4]. This would result in non-competitive inhibition kinetics. Note, however, that the K_i values reported in each case constitute the competitive-inhibition constants, since the kinetic method of analysis used here specifically accounts for transfer effects and represents interaction of an inhibitor with free enzyme.

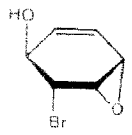
β-D-Galactosidase, which was initially treated with 3M sodium iodoacetate[5] in the presence of IPTG for 3 h and which was still fully active, was then reacted with **1** (after removal of the IPTG and the excess of sodium iodoacetate) for 25 min. This enzyme was subsequently irradiated with light at 350 nm. The light irradiation did not result in any additional loss of activity. The non-irradiated and the irradiated samples were dena-



4 R = OTs

5 R = SBz

6 R = SH



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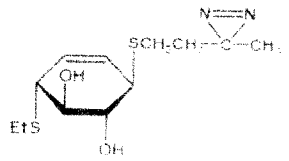

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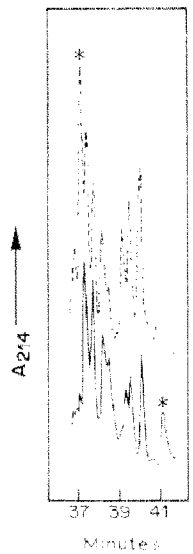

Fig. 2. Portions of the profiles of two h.p.l.c. elutions (~40 min after elution had started) showing distinct differences in peaks (differences are highlighted by*). [β-D-Galactosidase was treated with 3M sodium iodoacetate in the presence of 300mM IPTG for 3 h and then for 25 min with compound **1** after the IPTG and sodium iodoacetate was removed. The reaction components were again removed and one part of the solution was irradiated with light of 350 nm for 40 min. The enzyme was then denaturated with urea and carboxymethylated. After treatment with trypsin, the peptides were eluted by h.p.l.c. as described in the text. (———) not irradiated; (- - - - - -) irradiated].

tured in urea and carboxymethylated[6], and then treated with trypsin. The peptide elution-patterns from a reversed-phase h.p.l.c. column showed at least one distinct change in elution pattern (Fig. 2) showing that the diazirine end of **1** had reacted.

EXPERIMENTAL

General methods. — All reactions were monitored by t.l.c. on Silica Gel 60 F_{254} (Merck). Column chromatography was carried out with Silica 32-63, 60 A (ICN). Preparative h.p.l.c. separations involved Knauer components and a Hypersil column (250 × 20 mm, 5 μm, Bischoff). Analytical h.p.l.c. of the carboxymethylated trypsin peptides was carried out on a Vydac 218 TP (250 × 4.6mm) C_{18} reversed-phase column (1 mL/min). The gradient was as follows: 0 to 5 min: 0–10% MeCN in H_2O; 5 to 70 min: 10–45% MeCN in H_2O (in every case with 0.1% CF_3CO_2H). ^{1}H-N.m.r. spectra were recorded with a Bruker WM 250 spectrometer at 250 MHz as solutions in $CDCl_3$ (internal Me_4Si).

Enzymic reactions. — β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *E. coli* was purchased from Boehringer Mannheim. Isopropyl 1-thio-β-D-galactopyranoside, and *o*- and *p*-nitrophenyl β-D-galactopyranoside were obtained from Sigma. Assays were performed at 30° in 50mM sodium phosphate buffer (pH 6.8) containing mM magnesium chloride. For assays of the loss of activity as a function of time, enzyme (0.1 μg) was added to the assay solution (1 mL) containing 2mM ONPG. For determination of the inhibition constants enzyme (0.1 μg) was added to a range of concentrations of PNPG (0.01–0.15mM) and inhibitor (5–30mM) in the assay buffer. Units were defined as 1 μmol of nitrophenol produced per min under the assay conditions. Kinetic data were analyzed by the method of Deschavanne *et al.*[7] as modified by Huber and Gaunt[8], which takes into account the effect of the transfer reactions that can result from the reaction of β-D-galactosidase with inhibitors. The method gives K_i values that represent interaction of inhibitor with free enzyme.

S-(*3-Azibutyl) thiobenzoate* (**5**). — To 3-azibutyl *p*-toluenesulfonate[9] (**4**, 1.5 g, 5.9 mmol) in dry acetone (60 mL), potassium thiobenzoate (1.1 g, 6.2 mmol) was added and the solution was kept at room temperature for 3 h. The mixture was then concentrated to dryness, diluted with water (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was dried ($MgSO_4$), concentrated, and purified by column chromatography (1:10 EtOAc–cyclohexane) to give **5** (0.9 g, 70%) as a colourless syrup; R_F 0.40 (1:10 EtOAc–cyclohexane); ^{1}H-n.m.r.: δ 2.97 (t, 2 H, $J_{1,2}$ 7.7 Hz, H-1), 1.73 (t, 2 H, H-2), 1.11 (s, 3 H, H-4), 7.41–8.00 (m, 5 H, Ph).

(1,2/3,6)-*6-(3-Azibutylthio)-2-bromo-4-cyclohexene-1,3-diol* (**2**). — To compound **5** (1 g, 4.5 mmol) and compound **7** (1 g, 5.2 mmol) in dry EtOH (20 mL), methanolic M NaOMe was added dropwise to keep the solution basic until the reaction was complete (t.l.c.). The mixture was then desalted by eluting it through a silica gel column with MeOH. The eluate was concentrated and purified by h.p.l.c. (1:3 EtOAc–cyclohexane) to give syrupy **2** (1.25 g, 90%); R_F 0.09 (1:3 EtOAc–cyclohexane); λ_{max}

363nm, ^{1}H-n.m.r.: δ 4.09 (s, 1 H, $J_{1,2}$ 1.8, $J_{1,6}$ 5.3 Hz, H-1), 4.48 (dd, 1 H, $J_{2,3}$ 6.5 Hz, H-2), 4.54 (d, 1 H, H-3), 5.80 (s, 2 H, H-4,5), 3.43 (d, 1 H, H-6), 2.45 (t, 2 H, $J_{1',2'}$ 7.5 Hz, H-1'), 1.69 (t, 2 H, H-2'), 1.07 (s, 3 H, H-4'), 2.70–3.17 (m, 2 H, OH).

Anal. Calc. for $C_{10}H_{15}BrN_2O_2S$: C, 39.10; H, 4.92; N, 9.12. Found: C, 39.06; H, 4.76; N, 9.39.

(1*R*,2*S*,5*R*,6*S*)-*2-(3-Azibutylthio)-5,6-epoxy-3-cyclohexen-1-ol* (**1**). — To compound **2** (200 mg, 0.65 mmol) in dry CH_2Cl_2 (10 mL) powdered KOH (60 mg) and Et_4NBr (~4 mg) was added and the mixture was stirred for 5 min. The solid material was then centrifuged off and the clear solution was concentrated and used without further purification; R_F 0.34 (1:1 EtOAc–cyclohexane); λ_{max} 363nm.

(1*S*,3*R*,2,4)-*3-(3-Azibutylthio)-5-cyclohexene-1,2,4-triol* (**3**). — Compound **1** (100 mg, 0.325 mmol) was dissolved in water (1 mL) and kept at room temperature for 40 min. The solvent was then removed *in vacuo* and the residue purified by h.p.l.c. (2:1 EtOAc–cyclohexane, only absolutely pure fractions were collected) to give syrupy **3** (27 mg, 27%); R_F 0.16 (2:1 EtOAc–cyclohexane); λ_{max} 363 nm. For ^{1}H-n.m.r. analysis the product was acetylated with 1:2 Ac_2O–C_5H_5N (1 mL): δ 5.55 (ddt, 1 H, $J_{1,2}$ 7.8, $J_{1,4}$ 2.1, $J_{1,5}$ 2.1, $J_{1,6}$ 2.1 Hz, H-1), 5.15 (dd, 1 H, $J_{2,3}$ 11.7 Hz, H-2), 2.91 (dd, 1 H, $J_{3,4}$ 9.3 Hz, H-3), 5.49 (ddt, 1 H, $J_{4,5}$ 2.1, $J_{4,6}$ 2.1 Hz, H-4), 5.70 (dt, 1 H, $J_{5,6}$ 10.4 Hz, H-5), 5.78 (dt, 1 H, H-6), 2.45 (ddd, 1 H, $J_{1'a,1'b}$ 15.3, $J_{1'a,2'a}$ 6.8, $J_{1'a,2'b}$ 8.2 Hz, H-1'a), 2.47 (ddd, 1 H, $J_{1'b,2'a}$ 8.2, $J_{1'b,2'b}$ 6.8 Hz, H-1'b), 1.56 (ddd, 1 H, $J_{2'a,2'b}$ 15.0 Hz, H-2'a), 1.61 (ddd, 1 H, H-2'b), 1.05 (s, 3 H, H-4'), 2.07, 2.13, and 2.14 (3 × s, 9 H, OAc).

(1*S*,3*R*,2,6)-*3-(3-Azibutylthio-6-ethylthio-4-cyclohexene-1,2-diol* (**8**). — Ethanethiol (150 μL) and potassium *tert*-butoxide (~2 mg) were added to a stirred solution of compound **1** (100 mg, 0.325 mmol) in dry EtOH (1.5 mL). After 30 min at room temperature the mixture was concentrated and the residue roughly purified by column chromatography (1:2 EtOAc–cyclohexane). Further purification was performed by h.p.l.c. (2:5 EtOAc–cyclohexane). Only the pure centre fractions were collected and gave syrupy **8** (38 mg, 30%); R_F 0.19 (1:2 EtOAc–cyclohexane); ^{1}H-n.m.r.: δ 3.21–3.34 (m, 2 H, H-1,4), 3.55–3.68 (m, 2 H, H-2,3), 5.57–5.74 (2 × d, 2 H, $J_{5,6}$ 10.2 Hz, H-5,6), 2.37–2.48 (m, 2 H, $J_{1',2'}$ 7.5 Hz, H-1'), 1.68 (t, 2 H, H-2'), 1.06 (s, 1 H, H-4'), 2.59 (dq, 1 H, $J_{1''a,1''b}$ 12.0, $J_{1''a,2''}$ 7.5 Hz, H-1''a), 2.62 (dq, 1 H, $J_{1''b,2''}$ 7.5 Hz, H-1''b), 1.28 (t, 3 H, H-2'').

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