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Synthesis and Characterization of *cis*- and *trans*-2,3-Epoxybutane-1,4-diol 1,4-Bisphosphate, Potential Affinity Labels for Enzymes That Bind Sugar Bisphosphates[1,2,3]

JOHN V. SCHLOSS[4] AND FRED C. HARTMAN

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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cis- and *trans*-2,3-Epoxybutane-1,4-diol 1,4-bisphosphate, which can be considered reactive analogs of several sugar bisphosphates, have been synthesized in a continuing effort to develop new and diverse affinity labeling reagents for enzymes which bind phosphorylated substrates. *cis*-2,3-Epoxybutane-1,4-diol was obtained by epoxidation of commercially available *cis*-2-butene-1,4-diol with *m*-chloroperbenzoic acid; the trans epoxide was obtained by reduction of 2-butyne-1,4-diol with $LiAlH_4$ followed by epoxidation with *m*-chloroperbenzoic acid. The diols were phosphorylated with diphenyl chlorophosphate, and the phenyl blocking groups were then removed by Pt-catalyzed hydrogenation. By the criterion of their reaction with the sulfhydryl group of glutathione, the phosphorylated epoxides are 6000 times less electrophilic than the previously described and structurally similar reagent 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate.

A major advance in the study of protein structural–functional relationships has been the development of site-specific reagents or affinity labels (*1*). An affinity label is a chemically reactive compound which resembles the substrate of an enzyme and thus possesses affinity for the enzyme's active site; subsequent to reversible binding, the affinity label can covalently modify amino acid side-chains within the active site. Because of their high degree of specificity, affinity labels are powerful tools for the identification and mapping of the substrate-binding domain of enzymes and in some instances yield information about the mechanism of catalysis (*1*). Haloacetyl compounds (*2*) and haloketones (*3*) have found the broadest application as affinity labels, but various types of compounds, including epoxides, have been used with success (*1*).

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[4] Predoctoral fellow supported by Grant GM 1974 from the National Institutes of General Medical Sciences, National Institutes of Health. Current address: Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706.

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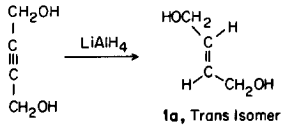
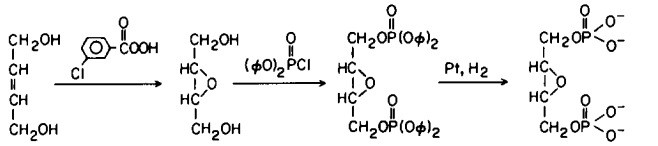


1a, Trans Isomer



1a, Trans Isomer b, Cis Isomer | 2a, Trans Isomer b, Cis Isomer | 3a, Trans Isomer b, Cis Isomer | 4a, Trans Isomer b, Cis Isomer

One of us recently reported the synthesis of the haloketone Br-butanone-P_2[5] (*4*). This reagent has proven to be an affinity label for ribulosebisphosphate carboxylase (EC 4.1.1.39), the enzyme responsible for photosynthetic fixation of CO_2 (*5, 6*). In an effort to find additional affinity labels for this and other enzymes that bind sugar bisphosphates, we have synthesized the cis and trans isomers of epoxybutane-P_2 according to Scheme I and have examined their stability and reactivity toward the cysteinyl residue (the most nucleophilic amino acid residue found in proteins) of the peptide glutathione (γ-L-glutamyl-L-cysteinylglycine). The interactions of the epoxides with several enzymes have also been examined.

EXPERIMENTAL PROCEDURES

Materials. *cis*-2-Butene-1,4-diol, *m*-chloroperbenzoic acid, and DTNB were obtained from Aldrich Chemical Co. 2-Butyne-1,4-diol and diphenyl chlorophosphate were purchased from Eastman Kodak. $LiAlH_4$ and platinum dioxide were products of Alfa Inorganics and Matthey Bishop, respectively. D-Ribulose 1,5-bisphosphate, 3-phosphoglycerate, glutathione, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and the diethylketal of glyceraldehyde-3-phosphate were purchased from Sigma Chemical Co. Fructose-1,6-bisphosphate and aldolase were purchased from Boehringer Mannheim. Alkaline phosphatase was obtained from Worthington. Ribulosebisphosphate carboxylase was purified as previously described (*7*).

General methods. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Melting points were determined with a Fisher–Johns apparatus and are uncorrected.

[5] Abbreviations used are: Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate; epoxybutane-P_2, *cis*- or *trans*-2,3-epoxybutane-1,4-diol 1,4-bisphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EDTA, ethylenediaminetetraacetic acid; tlc, thin-layer chromatography; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

nmr spectra were obtained with a 100-MHz Varian XL-100 nmr spectrometer. Spectra were recorded with samples dissolved in $CDCl_3$ or D_2O, and tetramethylsilane or DSS was used as an internal reference, respectively.

tlc was conducted on plastic sheets coated with silica gel or cellulose containing a fluorescent indicator (MN-Polygram Sil N-HR and Cel 300, sold by Brinkman Instruments, Inc.). The solvents used were diethyl ether (silica gel) and ethanol (cellulose). Descending paper chromatography was conducted by use of Whatman No. 1 paper with *n*-butyl alcohol/glacial acetic acid/water (7:2:5 v/v/v) as solvent. Ion-exchange paper chromatography was conducted by use of Whatman DE 81 paper with 0.4 *M* ammonium bicarbonate solvent. For detection of phosphate esters, chromatograms were sprayed with the ammonium molybdate reagent of Hanes and Isherwood (*8*), then exposed to uv light. Compounds containing vicinal hydroxyl groups were visualized with periodate-benzidine (*9*). The periodate spray was 0.05% (w/v) sodium metaperiodate rather than a saturated solution of the potassium salt as reported earlier. For detection of epoxides, chromatograms were sprayed with saturated aqueous LiCl/1% phenolphthalein in EtOH (1:1 v/v), then heated in an oven at 110°C for 5 min [this procedure selectively visualizes epoxides by detection of OH^- liberated upon opening of the oxirane by chloride; similar methods have commonly been used to estimate oxirane concentrations in solution (*10*)]. Epoxides appeared as red spots on a white background, and faded upon further heating. Epoxides were also detected with TNB (*11*). Iodine vapor was used to detect alkenes, alkynes, and epoxides.

Inorganic and total phosphate were assayed by the method of Ames and Dubin (*12*).

Ribulosebisphosphate carboxylase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and aldolase were assayed by published procedures (*5*, *13–15*).

Peracid was assayed by reduction with iodide and titration of the resulting iodine with sodium thiosulfate (*16*).

trans-2-Butene-1,4-diol (**1a**). To a 500-ml, 3-necked, round-bottomed flask equipped with stirrer, condenser, and dropping funnel were added 18 g (0.474 mol) of $LiAlH_4$ and 125 ml of tetrahydrofuran. The mixture was brought to reflux, and 10.2 g (0.118 mol) of 2-butyne-1,4-diol in 50 ml of tetrahydrofuran was added dropwise during 45 min. After it was boiled for 1 hr, the mixture was cooled to room temperature; 100 ml of 0.1 *N* HCl was then carefully added during 1 hr, and the mixture was boiled for an additional 15-min period. Anhydrous sodium sulfate (100 g) was added as a drying agent; it was removed by filtration through Celite and washed thoroughly with two 200-ml portions of dioxane. The combined filtrate and washings were treated with a second 100-g addition of Na_2SO_4. After Na_2SO_4 was removed, rotary evaporation of the filtrate gave 9.05 g (87%) of a viscous syrup. tlc (silica gel with diethylether as solvent) revealed virtually complete conversion of 2-butyne-1,4-diol (R_f 0.45) to **1a** R_f 0.20), which was well resolved from the commercially available cis isomer (**1b**) (R_f 0.27).

trans-2,3-Epoxybutane-1,4-diol (**2a**). To 3.62 g (0.0252 mol) of *m*-chloroperbenzoic acid in 50 ml of diethyl ether was added 2.11 g (0.0239 mol) of **1a**. After the

reaction mixture was incubated on ice in the dark for 2 hr, 67% of the peracid remained; incubation was continued for an additional 18 hr, at which time 17% remained. Crystals of **2a**, which had formed in the reaction mixture, were collected by filtration and washed with three 40-ml portions of cold diethyl ether to give 1.42 g (57%) of **2a**: mp 70–72°C [lit. mp 73.5–74.5°C (*17*)]; ^{1}H nmr (D_2O) δ 3.22 (m, 2 H, oxirane), 3.54 (dd, J_{gem} = 13 Hz, J_{vic} = 5.5 Hz, 2 H), 3.90 (dd, J_{vic} = 2.5 Hz, 2 H). *Anal.* Calcd for $C_4H_8O_3$ (104.11): C, 46.15; H, 7.75. Found: C, 46.23; H, 7.81. tlc (silica gel with diethyl ether as solvent) revealed a single component (R_f 0.15).

cis-2,3-Epoxybutane-1,4-diol (**2b**). To 20 g (0.116 mol) of *m*-chloroperbenzoic acid in 150 ml of diethyl ether was added 9.5 ml (0.115 mol) of **1b**. The reaction mixture was kept on ice in the dark; within 1 hr a copious precipitate had developed, and only 16% of the peracid remained. After an additional 2 hr, 11% of the peracid remained; the crystals of **2b** were collected by filtration and washed with cold diethyl ether to give 7.19 g (60%): mp 50–52°C; ^{1}H nmr (D_2O) δ 3.32 (m, 2 H, oxirane), 3.60 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2 H), 3.88 (dd, J_{vic} = 3.5 Hz, 2 H). *Anal.* Calcd for $C_4H_8O_3$ (104.11): C, 46.15; H, 7.75. Found: C, 46.24; H, 7.84. tlc (silica gel with diethyl ether as solvent) revealed a single component (R_f 0.19).

trans- and cis-2,3-Epoxybutane-1,4-diol 1,4-bis(diphenyl)phosphate (**3a,b**). To 1 g (9.6 mmol) of epoxide (**2a** or **2b**) in 5 ml pyridine was added 10 ml of $CHCl_3$. The mixture was cooled in an ice bath, and 6.1 ml (28.8 mmol) of diphenyl chlorophosphate was introduced. Phosphorylation was allowed to proceed at 4°C for 17 hr. Ice chips (2 g) were then added to the mixture, followed 3 hr later by 100 ml of $CHCl_3$. The $CHCl_3$ solution was washed successively with four 100-ml portions of 1 *N* H_2SO_4 and saturated aqueous $NaHCO_3$. After it was dried with Na_2SO_4, the $CHCl_3$ solution was concentrated to dryness to give either 5.32 g (98%) of the trans isomer (**3a**) or 4.84 g (89%) of the cis isomer (**3b**).

The trans isomer (**3a**) crystallized spontaneously on standing and was recrystallized twice from ethanol (70% recovery): mp 78–81°C; ^{1}H nmr ($CDCl_3$) δ 3.20 (t, 2 H, oxirane), 4.20 (dd, J_{gem} = 8 Hz, J_{vic} = 3 Hz, 2 H), 4.38 (dd, J_{vic} = 4 Hz, 2 H), 7.28 (m, 20 H, phenyl). *Anal.* Calcd for $C_{28}H_{26}O_9P_2$ (568.46): C, 59.16; H, 4.61; P, 10.90. Found: C, 59.26; H, 4.67; P, 10.91.

Both cis (R_f 0.58) and trans (R_f 0.47) isomers were homogeneous by tlc (silica gel with diethyl ether as solvent). The cis isomer (**3b**) had the following ^{1}H nmr ($CDCl_3$) spectra: δ 3.29 (t, 2 H, oxirane), 4.31 (m, 4 H), 7.21 (m, 20 H, phenyl).

trans- and cis-2,3-Epoxybutane-1,4-diol 1,4-bisphosphate tetralithium salt (**4a,b**). To 2 g (3.52 mmol) of **3a** or **3b** in 50 ml of anhydrous ethanol was added 2 g of activated charcoal (Darco, Atlas Powder Co.). Following filtration through Celite, the solution was subjected to Pt-catalyzed (100 mg of PtO_2) hydrogenation which was carried out for 1.5 hr at 52 psi in a Parr apparatus. After filtration through Celite, the solutions were chilled on ice and neutralized with 1.9 *N* LiOH in methanol. The precipitates were collected by centrifugation and washed twice with 200-ml portions of ethanol. After these were dried for 48 hr in a vacuum desicator, 0.84 g (78%) of the trans isomer or 0.79 g (73%) of the cis isomer (**4a** and **4b**) was recovered. Paper chromatography revealed that both compounds were homogeneous (R_f 0.14) except for a trace of inorganic phosphate (R_f 0.31) which

was removed by ion-exchange chromatography. After 0.5 g of **4a** or **4b** was dissolved in 50 ml of ice-cold water, the sample was applied to a refrigerated (4°C) column (2.5 × 59 cm) of Dowex 1-X2 [Cl^-] equilibrated with glass-distilled water. The column was eluted with a 2-liter linear gradient of 0–1 *M* LiCl; column elution and collection of samples were carried out at 4°C. Inorganic phosphate eluted at 0.35 *M* LiCl, while **4a** or **4b** eluted at 0.44 *M* LiCl. Pooled fractions containing **4a** or **4b** were concentrated at 27°C to 20 ml, and the phosphate ester was then precipitated by the addition of ethanol (200 ml). The precipitates were collected by centrifugation and washed twice with 200 ml of ethanol. After these were dried *in vacuo*, 0.39 g (78%) of **4a** or **4b** was obtained. The compounds were pure as judged by paper chromatography (R_f 0.14) or by ion-exchange paper chromatography (R_f 0.53).

Analysis of the trans isomer (**4a**) gave the following results: ^{1}H nmr (D_2O) δ 3.38 (m, 2 H, oxirane), 3.72 (dd, J_{gem} = 12 Hz, J_{vic} = 6 Hz, 2 H), 4.12 (dd, J_{vic} = 5.5 Hz, 2 H). *Anal.* Calcd for $C_4H_6O_9P_2Li_4 \cdot 2H_2O$ (323.83): C, 14.83; H, 3.11; P, 19.13; Li, 8.57. Found: C, 15.04; H, 3.20; P, 18.98; Li, 8.44.

Analysis of the cis isomer (**4b**) gave the following results: ^{1}H nmr (D_2O) δ 3.47 (m, 2 H, oxirane), 3.74 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2 H), 4.10 (dd, J_{vic} = 6 Hz, 2 H). *Anal.* Calcd for $C_4H_6O_9P_2Li_4 \cdot 3H_2O$ (341.85): C, 14.05; H, 3.54; P, 18.12; Li, 8.12. Found: C, 14.19; H, 3.42; P, 18.32; Li, 7.98.

The elemental analyses revealed the presence of <2% of 1 g-atom of chlorine in **4a** or **4b**, thereby demonstrating that insignificant opening of the epoxide ring by chloride ions has occurred.

RESULTS

Alkaline Phosphatase Treatment of Epoxybutane-P_2

Alkaline phosphatase (0.83 mg/ml) was incubated at room temperature with 0.05 *M* epoxybutane-P_2 (**4a** or **4b**) for 1.5 hr, at which time approximately 80% of the organic phosphate had been converted to inorganic phosphate. Inspection of the alkaline phosphatase-treated epoxybutane-P_2 by tlc (cellulose with ethanol as solvent) revealed the presence of the nonphosphorylated epoxides (R_f 0.92) and none of their hydrolysis products (threitol and erythritol). Chromatographic markers for threitol and erythritol (R_f 0.81) were obtained by base (1 *N* KOH, 1 hr, 100°C) or acid (1 *N* $HClO_4$, 1 hr, 100°C) hydrolysis of the nonphosphorylated epoxides (**2a** or **2b**). These results further verify the presence of the oxirane in **4a** or **4b** and suggest that little hydrolysis of the epoxide occurred during their preparation. Epoxybutane-P_2 could not be detected with the LiCl/phenolphthalein spray; even after alkaline phosphatase digestion, the resultant nonphosphorylated epoxides could not be detected prior to tlc. These observations suggest that phosphate esters and inorganic phosphate interfere with the detection of oxirane by reaction with halide. Consistent with this suggestion is our inability to estimate oxirane content of epoxybutane-P_2 in solution by use of various literature procedures which utilize similar detection methods (*10*).

Stability of Epoxybutane-P_2 in Aqueous Solution

When dissolved in D_2O, the cis and trans isomers have half-lives of approximately 8.0 and 8.5 hr at 24°C, respectively, as determined by the loss of the oxirane portion of their nmr spectra. After the compounds were incubated for 18 hr at 24°C, only 21% of the cis oxirane and 23% of the trans oxirane remained (Fig. 1); their respective nmr spectra substantially collapsed to multiplets centered at δ 3.86 and 3.91 (presumably threitol and erythritol bisphosphates). In contrast to the lack of stability of epoxybutane-P_2, the nonphosphorylated epoxides (**2a** and **2b**) were remarkably stable in solution. After 13 days at room temperature, no changes could be detected in the nmr spectra of **2a** or **2b** dissolved in D_2O.

Reaction of Epoxybutane-P_2 with Glutathione

Phosphorylated (**4a** and **4b**) or nonphosphorylated (**2a** or **2b**) epoxides (0.01 mmol) were dissolved in 0.95 ml of 0.5 *M* Bicine/1 m*M* EDTA (pH 8.0); to the

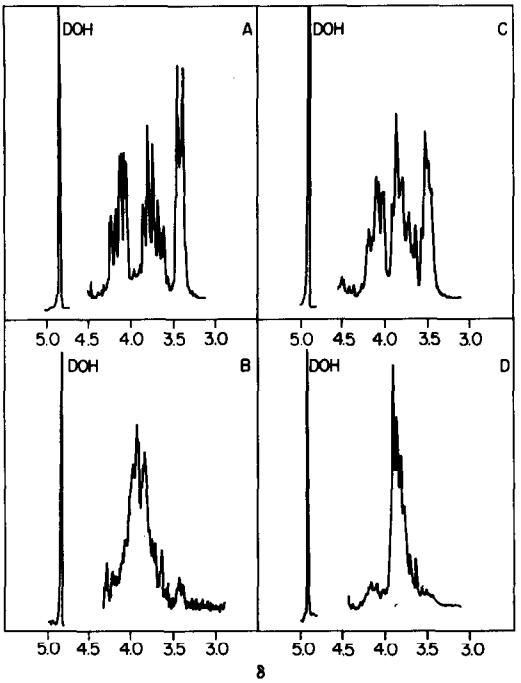


FIG. 1. ^1H nmr spectra of *cis*- and *trans*-epoxybutane-P_2 and their decomposition products. (A) *trans*-Epoxybutane-P_2 in D_2O relative to DSS: δ 3.38 (m, 2 H, oxirane), 3.72 (dd, J_{gem} = 12 Hz, J_{vic} = 6 Hz, 2 H), 4.12 (dd, J_{vic} = 5.5 Hz, 2 H). Fine splitting of the C-1 and C-4 hydrogen signals by the distal oxirane hydrogens can be observed with coupling constants of 2 and 3 Hz. (B) Same samples shown in (A) after 18 hr at 24°C. The spectrum has collapsed into a multiplet centered at δ 3.91, and the oxirane signal at δ 3.38 is greatly reduced. (C) *cis*-Epoxybutane-P_2 in D_2O relative to DSS: δ 3.47 (m, 2 H, oxirane), 3.74 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2 H), 4.10 (dd, J_{vic} = 6 Hz, 2 H). Fine splitting of the C-1 and C-4 hydrogen signals by the distal oxirane hydrogens can be observed with coupling constants of 2 and 3 Hz. (D) Same sample shown in (C) after 18 hr at 24°C. The spectrum has collapsed into a multiplet centered at δ 3.86, and the oxirane signal at δ 3.47 is greatly reduced.

solutions were added 0.05 ml of 0.1 *M* glutathione. The decrease in sulfhydryl concentration in reaction mixtures and controls (lacking epoxides) was monitored colorimetrically with DTNB (*18*). Periodically, 0.05-ml aliquots of mixtures were added to cuvettes containing 2.45 ml of 0.2 *M* potassium phosphate/0.4 m*M* DTNB (pH 8.0), and the subsequent increases in *A* at 412 nm were measured. By this criterion, second-order rate constants for the reaction of the epoxides with the free sulfhydryl group of glutathione were obtained. *cis*- and *trans*-Epoxybutane-P_2 gave values of 2.4×10^{-4} and 2.2×10^{-4} M^{-1} sec^{-1}, respectively, compared with 7.2×10^{-5} M^{-1} sec^{-1} for the nonphosphorylated epoxides. The previously reported (*4*) second-order rate constant for the reaction of Br-butanone-P_2 with glutathione (pH 8.0) is 1.4 M^{-1} sec^{-1}.

The reaction of the epoxides with glutathione was further characterized by inspection of products on the amino acid analyzer. Mixtures comprised of 0.4 *M* epoxide, 0.4 *M* glutathione, 1.6 *M* $KHCO_3$, and 1 m*M* EDTA (pH 8) were incubated for 20 hr; samples were diluted with 100 volumes of 0.066 *M* sodium citrate (pH 2.2), and 0.1-ml aliquots of the dilutions were chromatographed on the long column of a Beckman 120C amino acid analyzer. Prior to dilution of reaction mixtures of epoxybutane-P_2 with sodium citrate buffer, portions were digested with alkaline phosphatase (0.4 mg/ml) for 1.5 hr; these digests were also inspected on the amino acid analyzer. The results of the analyses are shown in Fig. 2. Control samples contained a mixture of glutathione and oxidized glutathione which eluted from the amino acid analyzer at 39 and 46 min, respectively. Reaction mixtures of nonphosphorylated epoxides (**2a** or **2b**) were devoid of glutathione and contained oxidized glutathione and a new ninhydrin-positive product which eluted from the analyzer at 23 min. Based on the ninhydrin color constant for oxidized glutathione, the latter product (presumably an *S*-alkylated glutathione) was formed in 72% yield. Reaction mixtures of *cis*- and *trans*-epoxybutane-P_2 also lacked glutathione and contained oxidized glutathione and an alkylation product (50% yield with the cis epoxide and 34% yield with the trans epoxide), which eluted at 14 min. The elevation of oxidized glutathione in reaction mixtures of epoxybutane-P_2 over that in controls or reaction mixtures of 2,3-epoxybutane-1,4-diol suggests that the phosphorylated epoxides increase the rate of oxidation of glutathione. Treatment of epoxybutane-P_2 reaction mixtures with alkaline phosphatase resulted in the disappearance of the compound that eluted from the analyzer at 14 min and the appearance of a corresponding amount of a new component at 23 min. The latter cochromatographed with the reaction product of glutathione and 2,3-epoxybutane-1,4-diol, thereby confirming that epoxybutane-P_2 and the corresponding nonphosphorylated epoxides react analogously with glutathione.

Effects of Epoxybutane-P_2 on Several Enzymes That Bind Bisphosphate Substrates

Several enzymes were examined for reversible inhibition and inactivation by epoxybutane-P_2: rabbit muscle fructosebisphosphate aldolase, yeast phosphoglycerate kinase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and

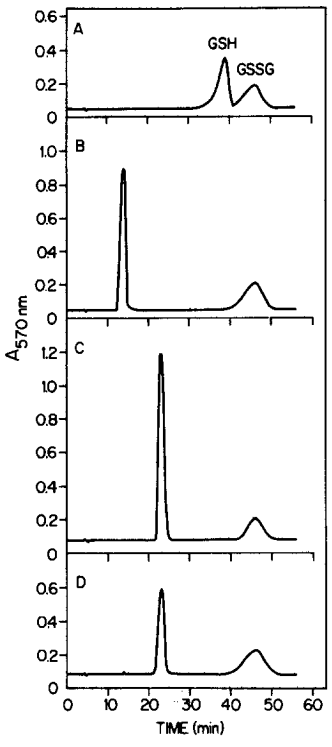


FIG. 2. Chromatographic profiles of glutathione, oxidized glutathione, and reaction products of glutathione on the long column of the amino acid analyzer. (A) Control (0.4 *M* glutathione) after a 20-hr incubation. (B) *cis*- or *trans*-Epoxybutane-P_2 (0.4 *M*) and glutathione (0.4 *M*) after a 20-hr incubation. (C) *cis*- or *trans*-2,3-Epoxybutane-1,4-diol (0.4 *M*) and glutathione (0.4 *M*) after a 20-hr incubation. (D) Same sample shown in (B) after treatment with alkaline phosphatase.

spinach ribulosebisphosphate carboxylase. Of these enzymes, ribulosebisphosphate carboxylase was of particular interest, since it is inactivated by the structurally related compound Br-butanone-P_2 in a fashion consistent with affinity labeling (*5*). Epoxybutane-P_2 (10 m*M* cis or trans isomer) did not inactivate ribulosebisphosphate carboxylase when incubated with the enzyme at room temperature for 19 hr [incubations were conducted in 0.05 *M* Bicine, 0.066 *M* $NaHCO_3$, 5 m*M* $MgCl_2$ (pH 8.0)]. Furthermore, in contrast to the haloketone, which also inhibits the enzyme competitively (K_I of 1.0 m*M* with respect to the substrate ribulosebisphosphate), *cis*- and *trans*-epoxybutane-P_2 are noncompetitive inhibitors of ribulosebisphosphate carboxylase. The apparent dissociation constants for *cis*- and *trans*-epoxybutane-P_2 · enzyme complex based on slope effects seen in Lineweaver–Burke plots (*19*) (enzymic reaction velocity^{-1} vs substrate concentration^{-1}) are 6.6 and 5.4 m*M* (K_{IS}), respectively, while their respective dissociation constants based on intercept effects are 2.0 and 2.1 m*M* (K_{II}) [see citation in Ref. (*20*) for definitions of terms]. Thus, unlike the haloketone, the epoxides not only fail to inactivate the enzyme but may bind to a different site on the enzyme as well.

The other enzymes tested also proved to be resistant to inactivation by 10 m*M* epoxybutane-P_2 (either isomer) when incubated with reagent for 10 hr under conditions similar to those used to assay the given enzyme. However, all three enzymes were inhibited reversibly in a competitive fashion by both *cis*- and *trans*-epoxybutane-P_2. The apparent dissociation constants for the epoxybutane-P_2 · enzyme complex (K_I) for the cis and trans isomers, respectively, are as follows: aldolase, 0.11 and 0.12 m*M*; phosphoglycerate kinase, 4.3 and 2.1 m*M*; glyceraldehyde-3-phosphate dehydrogenase, 7.1 and 8.1 m*M*. The K_I values obtained for epoxybutane-P_2 inhibition of aldolase are similar to those of other bisphosphate esters (*21*).

DISCUSSION

The overall yields of pure *trans*- and *cis*-epoxybutane-P_2 from the commercially available starting materials 2-butyne-1,4-diol and *cis*-2-butene-1,4-diol were 19 and 33%, respectively. The crystalline epoxide intermediates **2a** and **2b** can easily be prepared in large quantity and stored at −20°C for more than a year without sign of decomposition (as judged by their nmr spectra). These epoxides can also be stored at room temperature in aqueous (D_2O) solution for up to 2 weeks without decomposition. However, the crystalline cis isomer (**2b**) decomposes to a viscous syrup within several weeks at room temperature.

cis- and *trans*-Epoxybutane-P_2 were freed of trace contamination of inorganic phosphate, which resulted from hydrogenation of their immediate precursors **3b** and **3a**, by ion-exchange chromatography on Dowex-1 at low temperature (4°C). Use of the conditions previously described for the purification of the diethyl ketal of Br-butanone-P_2 (*4*) (chromatography at room temperature under slightly acidic conditions) results in decomposition of epoxybutane-P_2. Similar to the unphosphorylated epoxides, the crystalline tetralithium salts of epoxybutane-P_2 can be stored at −20°C indefinitely. However, in solution *trans*- and *cis*-epoxybutane-P_2 are unstable, with half-lives (D_2O, 24°C) of 8.0 and 8.5 hr, respectively.

Several lines of evidence confirm the assigned structures of *cis*- and *trans*-epoxybutane-P_2. (1) Their elemental analyses agree with theory. (2) Their nmr spectra are different (consistent with two different isomers) and contain an oxirane signal which is lost upon extended incubation in solution. The nmr spectra of the nonphosphorylated epoxides (**2a** and **2b**) are very similar to those of *trans*- and *cis*-epoxybutane-P_2. (3) The isomers of epoxybutane-P_2 chromatograph as single components on paper or ion-exchange paper in positions expected for bisphosphate esters. (4) After epoxybutane-P_2 is treated with alkaline phosphatase, the nonphosphorylated epoxides (but not their corresponding tetritols) can be detected by tlc. (5) Both isomers are electrophilic as judged by their reaction with the sulfhydryl groups of glutathione and TNB. (6) The enzyme aldolase has an affinity for *cis*- and *trans*-epoxybutane-P_2 (K_I = 0.11 and 0.12 m*M*, respectively) which is similar to that for various other bisphosphate esters (*21*).

Br-butanone-P_2 reacts with the sulfhydryl of glutathione approximately 6000 times more rapidly than epoxybutane-P_2. As mentioned earlier, Br-butanone-P_2 is

an affinity label for ribulosebisphosphate carboxylase and inactivates the enzyme in a time-dependent fashion. The lack of inactivation of this enzyme by *cis*- and *trans*-epoxybutane-P_2 may be due to their greatly reduced electrophilicity or to the fact that the enzyme apparently binds these bisphosphate esters differently than Br-butanone-P_2 (*cis*- and *trans*-epoxybutane-P_2 are noncompetitive inhibitors with respect to the substrate ribulosebisphosphate, whereas Br-butanone-P_2 is a competitive inhibitor).

The weak electrophilicity of epoxybutane-P_2 presents both advantages and disadvantages for a potential affinity labeling reagent. Because of its sluggish reactivity, epoxybutane-P_2 will probably prove less versatile than haloacetyl or haloketone affinity labels. Reactive substrate analogs containing halogen as the leaving group are frequently suitable as "general affinity labels" for different enzymes having similar specificities in binding (*22*). An advantage of reduced reactivity should be less nonspecific modification of any enzyme that is susceptible to inactivation in comparison to that seen with the more reactive reagents (see results obtained with Br-butanone-P_2) (*5*).

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