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Synthesis of (5,6)-epoxy-(1α, 2β, 3α, 4β)-(±)-5-cyclohexane- 1-^{3}H , 2,3, 4-^{3}H -tetrol. (^{3}H -Conduritol B epoxide).

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SUMMARY

Tritium labeled Conduritol B epoxide was prepared by a six step synthesis, starting with p-benzoquinone. The tritium was introduced into the molecule in the second step by reducing the quinone with sodium boro ^{3}H hydride.

Key words: Conduritol B, conduritol B epoxide, reduction with sodium boro-tritiate, Gaucher's disease, glucocerebrosidase, affinity labeling of the enzymes active side.

INTRODUCTION

Gaucher's disease is an inherited autosomal disorder characterized by a deficiency of the enzyme glucosyl ceramide β-glucosidase (1). This lysosomal enzyme cleaves the glycosidic bond of glucocerebroside to yield glucose and ceramide. In patients with Gaucher's disease an accumulation of glucocerebroside is found in various organs. The discovery by Legler (2) that conduritol B epoxide

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The abbreviations used are: CBE, conduritol B epoxide; PPY, 4-pyrrolidinopyridine same as 4-(1-pyrrolidinyl) pyridine.

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irreversibly inhibited the activities of β-glucosidases of differing origins, led to the introduction of a mouse model for Gaucher's disease (3). Conduritol B epoxide exerts its effect by acting as an analogue of the glucose moiety of the natural substrate, glucocerebroside. The enzyme reacts with the epoxide ring of CBE, binding covalently to the inhibitor, thus destroying enzyme activity irreversibly. Radiolabeled CBE has been used to study the distribution of CBE in vivo in the Gaucher mouse (4) and as an affinity label for the active site of the enzyme (5). CBE was first described by Nakajima et al. (6) and labeled with ^{14}C or ^{3}H by Legler (7) using a synthetic scheme starting with radiolabeled myoinositol. The method produces a number of isomers which have to be separated by paper chromatography, resulting in a low yield of the desired labeled CBE. Quaroni et al. (8) used the Wilzbach (9) technique to label CBE. However, the product obtained had a low specific activity.

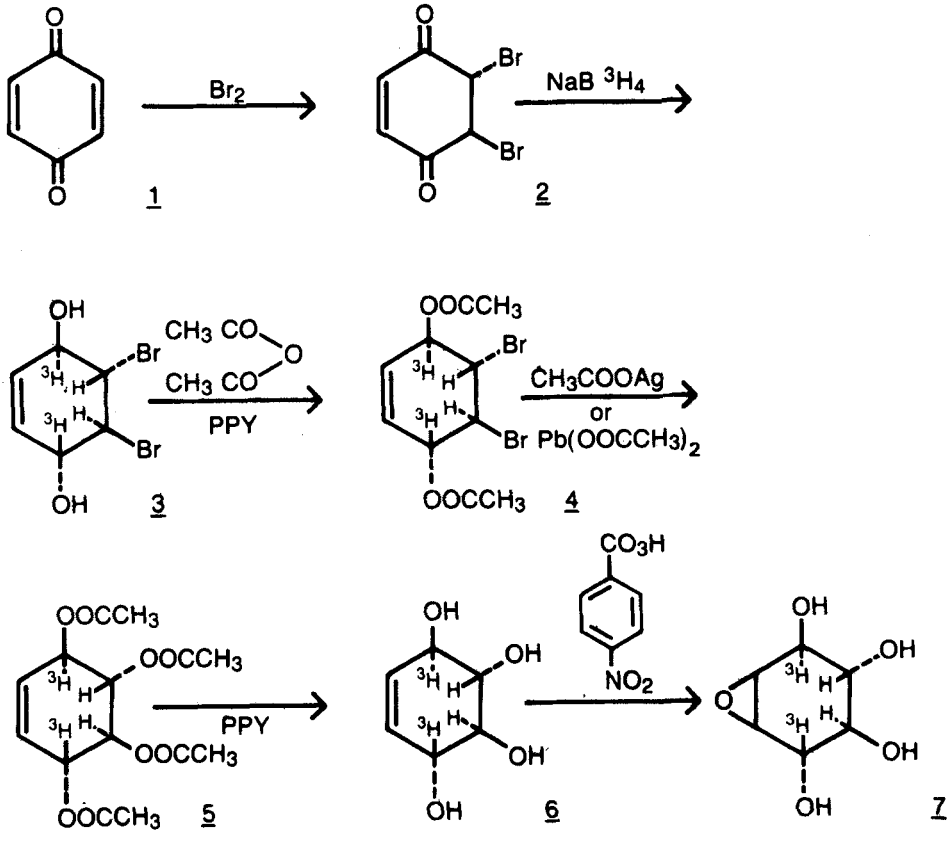
In the present paper a method is described for the synthesis of tritium labeled conduritol B epoxide, for the production of the compound with high specific activity. The synthetic scheme (see scheme), consists of six steps starting with p-benzoquinone (1), which when brominated yields (±)-trans-5, 6-dibromo-2-cyclohexene-1,4-dione (2). Stegelmeier (10) reduced this compound using sodium borohydride to yield (1α, 2β, 3α, 4β)-(±)-2,3-dibromo-1,4-dihydroxy-5-cyclohexene (3), which has all its ring substituents in the same *trans equatorial* configuration as conduritol B (6). Conduritol B was obtained by him by acetylation of (3) followed by exchange of the bromines with acetoxy groups, and then deacetylation. A modified approach is presented in this paper for the synthesis of tritiated conduritol B using tritiated sodium borohydride for the incorporation of tritium into the final product. After epoxidation of labeled conduritol B (6), radiolabeled conduritol B epoxide (7) was obtained.

EXPERIMENTAL

Materials and Methods

Silica gel 60 (E.Merck) plates were used for thin-layer chromatography. After development, the compounds were visualized by charring with an ammonium bisulfate solution (30% w/v) at 220°C for 20 min (11) with sulfuric acid-ethanol

SCHEME



(10% v/v) (12) or with the Dragendorff's reagent (13). p-Benzoquinone, sodium borohydride and lead II acetate were purchased from Aldrich Chemical Co. 4-Pyrrolidinopyridine (PPY) was obtained from Sigma Chemical Co. 4-Nitroperbenzoic acid was bought from Chemical Dynamics Corporation. 4.4-Thiobis-(6-t-butyl-3-methyl phenol) was purchased from K & K Laboratories, Division of ICN Biochemicals Inc. Tritiated sodium borohydride (25 mCi, 2-400 mCi/mmol) was purchased from NEN Research Products.

Radioactive measurements were carried out in a Searle Mark IV liquid scintillation counter using Aquasol (NEN Research Products). Radioscans were made using a Berthold LB 2842 automatic TLC-linear analyzer.

Melting points were taken with a Thomas-Hoover melting point apparatus and are reported corrected. Microanalyses were made by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

(±)-Trans-5,6-dibromo-2-cyclohexene-1,4-dione (2).

Bromine (16 g, 0.1 mol) in 150 ml of carbon tetrachloride was added dropwise over 45 minutes at 0°C to a stirred solution of p-benzoquinone (1) (10.8 g, 0.1 mol) in 250 ml of carbon tetrachloride. The solution was kept at this temperature for 30 minutes and then allowed to warm to room temperature. The solution was washed twice with 200 ml of water, dried over sodium sulfate and evaporated. The residue was dissolved in 500 ml of carbon tetrachloride and kept at 4°C for two days. A precipitated byproduct which melted at 186-187°C was filtered and the filtrate reduced to one fifth of its original volume. An equal volume of hexane was added and upon cooling to 4°C, the product precipitated to yield 17.5 g (65%) of trans-5,6-dibromo-2-cyclohexene-1,4-dione; mp 86.5-87.5°C.

(1α, 2 β, 3α, 4β)-(±)-2,3-Dibromo-[1,4-^{3}H]-dihydroxy-5-cyclohexene (3).

(a) Sodium boro[^{3}H]hydride (189 mg, 5 mmol, 50 mCi) in 5 ml of water was cooled to 0°C and added over the course of one hour, while stirring vigorously, to a solution of 5,6-dibromo-2-cyclohexene-1,4-dione (2) (1.07 g, 4 mmol) in 50 ml of diethyl ether at 0°C. After 15 min, the temperature was raised and kept at 25°C for one hour. The phases were separated and the aqueous phase was washed with 30 ml of ether. The ether phase was dried over sodium sulfate and evaporated. To eliminate any labile tritium, the residue was dissolved in 50 ml of ethanol and the solution was evaporated. This procedure was repeated three times. The residue was recrystallized twice from 19 ml of benzene yielding 653 mg (60%) of (3), mp 155-156° (Lit.(10) 149° C). Thin-layer chromatography in benzene-methanol (9:1) gave and R_f value for (3) of 0.4. The specific activity of the product was 2.1 mCi/mmol. Anal. Calcd. for $C_6H_8Br_2O_2$: C, 26.50; H, 2.96; Br, 58.77. Found: C, 26.98; H, 3.13; Br, 58.14.

(b) The method described above was repeated with sodium boro[^{3}H]hydride (47 mg, 1.25 mmol, 50 mCi) and using only 1 mmol of the dibromo cyclohexene dione. The specific activity of the product obtained was 9.6 mCi/mmol.

(c) A solution of sodium boro[^{3}H]hydride (37.8 mg, 1 mmol, 25 mCi) in 3 ml of water was cooled to 0°C. Over the course of one hour, this solution was added to a solution of (2) (536 mg, 2 mmol) in 25 ml of diethyl ether at 0°C. The reaction was allowed to proceed for 16 hr at 0°C after which cold sodium borohydride (114 mg, 3 mmol) in 1 ml of water was added over the course of one hour. The reaction mixture was warmed to 25°C and kept at this temperature for an additional hour. It was then worked up as described above, yielding 295 mg (54%) of (3) mp 148-154°C. The specific activity of the product was 2 mCi/mmol.

(1α, 2β, 3α, 4β)-(±)-2,3-Dibromo[1,4-^{3}H]-diacetyl-5-cyclohexene (4).

2,3-Dibromo-[1,4-^{3}H]-dihydroxy-5-cyclohexene (544 mg, 2 mmol), 4-pyrrolidinopyridine (296 mg, 2 mmol) and 6 ml of acetic anhydride were dissolved in 40 ml of methylene chloride. After three days at room temperature, the solvents were evaporated. The residue was dissolved in 40 ml of methylene chloride and washed with 3 ml of 1 N hydrochloric acid and 3 ml of water. The organic phase was dried over sodium sulfate and evaporated. The residue was recrystallized from 4 ml of isopropanol yielding 524 mg (74%) of (4) mp 93-94°C. Lit. (10) 94°C. Thin layer chromatography in benzene-methanol (95:5) gave an R_f value for (4) of 0.9. In a solvent system of benzene-chloroform-ethyl acetate (7:3:1), the R_f value was 0.6. The specific activity of the product was 2.1 mCi/mmol.

(1 α, 2 β, 3 α, 4 β)-(±)-[1-^{3}H], 2,3, [4-^{3}H]-Tetracetyl-5-cyclohexene (5). [^{3}H]-Conduritol B tetraacetate.

(a) Using silver acetate.

In a 10 ml ampoule were placed 2,3-Dibromo-[1,4-H]-diacetyl-5-cyclohexene (4) (356 mg, 1 mmol), silver acetate (626 mg, 3.8 mmol), 5 ml of acetic acid, 1 ml of acetic anhydride, and a stirring bar. The ampoule was sealed and the mixtured stirred and heated at 120°C for 18 hr in the dark. The contents of the ampoule were filtered and the silver salts were washed with 5 ml of acetic acid. Acetic acid and anhydride were distilled finally in high vacuum and the remaining product was dissolved in diethyl ether. The etheral solution was filtered and evaporated. The residue was recrystallized from 2 ml of isopropyl ether yielding 214 mg (68%) (308 mg) of (5) mp 85-86°C. Lit. (14) 85.5-86°C.

Thin-layer chromatography in benzene-methanol (95:5) gave an R_f value for this compound of 0.8. In a solvent system of benzene-chloroform-ethyl acetate (7:3:1), the R_f value was 0.25. The specific activity of the product was 2 mCi/mmol.

(b) Using anhydrous lead II acetate.

A mixture of 2,3-dibromo-[1,4-^{3}H]-diacetyl-5-cyclohexene (4) (356 mg, 1 mmol), anhydrous lead II acetate (650 mg, 2 mmol), 10 ml of acetic anhydride and 8 ml of acetic acid were refluxed at 130°C for one day. An additional 650 mg of lead acetate was added and refluxing continued for an additional day. Acetic acid and anhydride were removed under reduced pressure and the residue was suspended in diethyl ether. The suspension was filtered and the ether was evaporated. The residue was recrystallized from 3 ml of isopropyl ether yielding 170 mg (54%) of (5) mp 85-86°C. The specific activity of the product was 2 mCi/mmol.

(1α, 2β, 3α, 4β)-(±)-5-Cyclohexane-[1-^{3}H],2,3,[4-^{3}H]-tetrol (6). [^{3}H] Conduritol B.

To a solution of [^{3}H]-Conduritol B tetraacetate (157 mg, 0.5 mmol) in 5 ml of methanol, 4-pyrrolidinopyridine (74 mg, 0.5 mmol) was added. The mixture was allowed to stand at room temperature for two days after which the solvent was evaporated. The residue was suspended in 10 ml of acetone and stirred for one day. The product was filtered and washed with 5 ml of acetone yielding 66 mg (91%) of Conduritol B. mp 202-203°C. Lit. (15) 204.5-205°C. The product can be recrystallized from methanol (50x v/w). Thin-layer chromatography in hexane-isopropanol-water (1:2, 10%) gave an R_f value of 0.65. The spots were visualized with ammonium bisulfate. Dragendorff reagent shows only a faint spot. The specific activity of this compound was 2 mCi/mmol.

(5,6)-Epoxy-(1α, 2β, 3α, 4β)-(±)-5-cyclohexane-[1-^{3}H],2,3,[4-^{3}H]-tetrol (7). [^{3}H]-Conduritol B Epoxide (6).

[^{3}H]-Conduritol B (6) (73 mg, 0.5 mmol) was dissolved in 11 ml of methanol, then p-nitroperbenzoic acid (275 mg, 1.5 mmol) was added to the clear solution. It was allowed to stand at room temperature for three days after which the meth-

anol was evaporated. The residue was suspended in 10 ml of acetone and the mixture was stirred for 16 hr. The product was filtered and washed with 10 ml of acetone, yielding 69 mg (85%) of [^{3}H]-Conduritol B epoxide (6) mp 161-162°C. Lit. (14) 159-161°C. For further purification the product was dissolved in 2 volumes/ weight of water and precipated by the addition of 20 volumes of ethanol, yielding (7) melting at 165-166°C. Thin-layer chromatography in hexane-isopropanol-water (1:2, 10%) gave an R_f of 0.5. The epoxide could be visualized by spraying with Dragendorff's reagent. Both Conduritol B and Conduritol B epoxide could be detected if the TLC plates were sprayed first with the Dragendorff's reagent and dried, followed by spraying with the ammonium bisulfate solution. Alternatively both compounds could be detected simultaneously by combining the two spray reagents (Dragendorff's reagent-ammonium bisulfate (1:3)). More epoxide could be isolated from the acetone solution by evaporating it and partitioning the residue between diethyl ether and water. Lyophilization of the aqueous solution yielded about 12% more of the product. The specific activity of the [^{3}H]-conduritol B epoxide was 2 mCi/mmol.

Preparative thin-layer chromatography was used to purify (7) in preparations where [^{3}H]-Conduritol B remained as an impurity. The impure product (3 mg) was spotted on E.M. Merck, Silica Gel 60 plates (0.5 mm) and the plates were developed as described above. The radioactive bands were visualized after five days exposure to Kodak X-Omatic AR film. The band corresponding to the radioactive CBE was scraped off and eluted with 25 ml of chloroform-methanol-water (5:10:4) (16) yielding 2.3 mg of [^{3}H]-Conduritol B epoxide. Radioscans of this product showed only a single radioactive peak.

DISCUSSION

The bromination of benzoquinone, as found by Nef (17), leads to the formation of trans-5,6-dibromo-2-cyclohexene-1,4-dione (2). This compound was also prepared by Savoie and Brassard (18) and later by Stegelmeier (10) who published an erroneous melting point of 96°C, for this compound. Repeated recrystallizations wouldn't change the mp from 86.5-87.5°C observed also by Norris and Sternhell (19). The method described in this paper is a modification of the

published procedures. A byproduct of this reaction was probably 2,5-dibromohydroquinone described with the same melting point by Billman et al. (20).

By reducing this dione to the diol with sodium boro[^{3}H]hydride we obtained (1α, 2β, 3α, 4β)-(±)-2,3-dibromo-[1,4-^{3}H]-dihydroxy-5-cyclohexene (3). Theoretically the reduction of a dione to a diol should be possible using 0.5 mole of sodium borohydride per mole of dione. It was found in the course of this work that by using less than 1.25 mole of sodium borohydride per mole of dione, satisfactory chemical yields of the diol could not be obtained. Half of the radioactivity of the tritiated diol is lost through solvent interaction (21) and another half by the exchange of labile tritium atoms, thereby reducing the radioactive yield to only about 25%. One approach attempted to increase the radioactive and chemical yields was to first reduce (2) with 0.5 mole of sodium boro [^{3}H] hydride per mole (2). This reaction was allowed to proceed slowly at 0°C, and then completed using an excess of "cold" sodium borohydride. However, neither the chemical nor the radioactive yields of the product could be improved. The analytically pure dibromo diol (3) melted at 155°C, six degrees higher than reported in the literature (10). The labile tritium could be almost completely exchanged by co-distillation with ethanol. The remaining traces were eliminated in subsequent steps and recrystallizations. The tritium labeled 2,3-dibromo-1,4-dihydroxy-5-cyclohexene (3) was acylated using 4-pyrrolidinopyridine as the catalyst. This acylation catalyst was described by Hofle, et al. (22) as being 10^4 times more active than pyridine. An equimolar amount of the catalyst was used although preliminary experiments indicated that half of this amount could be used. The lower limit of the ratio of catalyst to reactants was not explored.

In the synthesis of Conduritol B tetraacetate (5) Stegelmeier (10) replaced the bromine groups with acetyl groups using silver acetate in refluxing acetic acid. For the synthesis of radiolabeled (5) it was desirable to develop a method which should be applicable for a small scale reaction. Thus, the reaction was performed in a sealed ampoule, with a sealed in stirring bar, avoiding the difficulties related to the refluxing of a small volume of acetic acid, as well as allowing for the reaction to be carried out at temperatures higher

than the boiling point of the solvent. In this work another simpler and more convenient method was developed by using anhydrous lead II acetate for the acetylation by modifying a method described by Shank and Eistert (23). This reagent being soluble in acetic acid would allow for the reaction to be carried out as a homogenous solution rather than a suspension. Good yields were obtained with an excess of acetic anhydride at 130°C. Thin-layer chromatography showed no more starting material.

Conduritol B (6), originally synthesized by McCasland and Horswill (24), was obtained by Legler (2) by hydrolizing conduritol B tetraacetate (5) according to the method of Zemplen (25,26) using a catalytic amount of sodium methoxide and by Radin and Vunnam (14) who used triethylamine. As described in this paper, it was found that 4-pyrrolidinopyridine, an excellent acylation catalyst, could also be used for deacetylation. This compound is very soluble in acetone so it is easy to separate the almost insoluble tritiated conduritol B (6) in satisfactory yield after the reaction.

Conduritol B epoxide (7) was first synthesized by Nakajima, et al (6) who used perbenzoic acid in chloroform and acetic acid for the epoxidation of Conduritol B. Leger (7) used p-nitroperbenzoic acid in acetic acid, even though Conduritol B is not soluble in this acid; the reaction was performed as a suspension. Radin and Vunnam used m-chloroperbenzoic acid in methanol to epoxidize Conduritol B (14). By repeating these methods we could not obtain the epoxide without contamination from Conduritol B. To improve the yield and purity of Conduritol B epoxide, several methods were tried unsuccessfully: The addition of platinic oxide (0.1%) inhibited the epoxidation.

Elevation of the reaction temperature diminished the yield even by using the antioxydant 4,4'-thiobis-(6-t-butyl-3-methylphenol) which according to Kishi et al. (27) stabilizes the peracid (for 3 hours, even at 90° C).

The method developed during this work uses an excess of p-nitroperbenzoic acid (minimum three times the theoretical amount). This reaction, in dilute methanolic solution and lasting several days, resulted in a product which showed only a single spot on thin-layer chromatography. Some preparations showed the presence of traces of the starting material, radiolabeled Conduritol B, as indi-

cated by the radioscans of plates which were developed by thin-layer chromatography. In order to obtain a radiochemically pure product, purification by quantitative layer chromatography was therefore used. The synthesis described in this paper was made on a 2 mCi/mmol level. It was repeated with sodium boro[^{3}H]hydride with four times higher specific activity resulting in tritiated CBE with a specific activity of about 10 mCi/mmol.

The synthesis described in this paper would allow the preparation of labeled conduritol B epoxide with considerably higher specific activity.

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