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OXIDATION OF BIPHENYL BY A BEIJERINCKIA SPECIES

David T. Gibson, Rowena L. Roberts, Martha C. Wells and Val M. Kobal

Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712

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SUMMARY

A species of Beijerinckia that utilizes biphenyl as sole source of carbon for growth was isolated by enrichment culture. A mutant strain, Beijerinckia B8/36, oxidizes biphenyl to cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene. Cell extracts, prepared from the parent organism, oxidize cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene to 2,3-dihydroxybiphenyl. The physical properties of both metabolites are described.

INTRODUCTION

Polychlorinated biphenyls are worldwide environmental pollutants (1). Relatively little is known about the ability of microorganisms to degrade biphenyl and its 210 possible chlorinated isomers. Lunt and Evans reported that certain Gram-negative bacteria degraded biphenyl through 2,3-dihydroxybiphenyl, α-hydroxy-δ-phenylmuconic semialdehyde and phenylpyruvate (2). Pseudomonas putida was reported to oxidize biphenyl through 2,3-dihydro-2,3-dihydroxybiphenyl and benzoic acid (3). The identification of 2,3-dihydro-2,3-dihydroxybiphenyl was inferred by acid-catalyzed dehydration of a metabolite to give a mixture of 2- and 3-hydroxybiphenyl. In this communication we report the isolation and identification of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene [cis-2,3-dihydro-2,3-dihydroxybiphenyl] and 2,3-dihydroxybiphenyl. Both compounds are intermediates in the degradation of biphenyl by a strain of Beijerinckia.

MATERIALS AND METHODS

Beijerinckia [wild-type] and Beijerinckia B8/36 were grown in mineral salts medium (4) containing 0.2% succinate and 0.1% biphenyl. Beijerinckia B8/36 was obtained by treatment of the parent strain with N-methyl-N'-

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nitro-N-nitrosoguanidine as described previously (5). Ten liter cultures were grown in a New Brunswick Model M14 Microferm fermentor. Cell extracts were prepared by sonication.

Isolation of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene. Beijerinckia B8/36 was grown in 10 liters of the above medium. After 5 hours the cells were removed by centrifugation and the clear supernatant solution was extracted with 3 liters of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and the solvent removed to leave 4.6 g of a solid residue.

Acid catalyzed dehydration. cis-2,3-Dihydroxy-1-phenylcyclohexa-4,6-diene [10 mg] was dissolved in 3.0 ml of diethylether. The reaction was initiated by the addition of 10 µl of concentrated HCl. After 30 minutes the ether solution was analyzed by gas chromatography in a Varian Aerograph modal 90-P with a thermal conductivity detector [He: 60 ml/min]. The column [1/4" × 5'] was packed with 3% SE 30 on Diatomite CLQ. Temperatures used were: column 210°, injector 210° and detector 265°. Under these conditions two products were obtained that showed retention times of 2.33 and 3.33 minutes, respectively. Under the same conditions synthetic samples of 2- and 3-hydroxybiphenyl also showed retention times of 2.33 and 3.33 minutes, respectively. The amounts of 2-hydroxybiphenyl [66%] and 3-hydroxybiphenyl [34%] formed from cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene were determined by automatic integration of the area under each peak with reference to standard amounts of synthetic samples. In a separate experiment the dehydration products from 100 mg of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene were separated by preparative thin layer chromatography on silica gel. The solvent used was benzene:methanol [27:3]. The areas on the plate that contained 2- and 3-hydroxybiphenyl were removed and extracted with ether. Removal of the solvent from each extract gave 2-hydroxybiphenyl [47 mg] and 3-hydroxybiphenyl [17 mg].

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Preparation of 8,9-diacetoxy-1-phenyl-4-[p-bromophenyl]-2,4,6-triazatricyclo[5.2.2.$0^{2,6}$]undec-10-ene-3,5-diene. cis-2,3-Dihydroxy-1-phenylcyclohexa-4,6-diene [10 mmoles] was dissolved in 22 mmoles of acetic anhydride. Pyridine [2.0 ml] was added and the mixture was left at 0° for 24 hours. At this time the reaction mixture was poured into 25 mls of ether:water [1:1], and shaken twice with a saturated solution of $CuSO_4$. The ether layer was washed with water and dried over anhydrous Na_2SO_4. Removal of the solvent gave an oily residue that was dissolved in 2.0 mls of acetone. Freshly sublimed 4-[p-bromophenyl]-1,2,4-triazoline-3,5-dione* [9.3 mmoles] was added to the solution. The reaction was performed at 0° in the dark. After 10 minutes small amounts of the dienophile were added until a red color persisted in the reaction mixture. The solvent was removed in vacuo and the residue recrystallized twice from acetone-hexane.

Preparation of [Z]-3a,7a-Dihydro-4-phenyl-2,2-dimethyl-1,3-benzodioxole. cis-2,3-Dihydroxy-1-phenylcyclohexa-4,6-diene [100 mg] was dissolved in 5.0 ml of 2,2-dimethoxypropane. The solution was cooled in ice water and 10 µl of 5N HCl was added from a Hamilton syringe. After 30 minutes the solvent was removed and the residue applied to a column [1 × 40 cm] of basic alumina. Elution with chloroform gave 36 mg of pure [Z]-3a,7a-dihydro-4-phenyl-2,2-dimethyl-1,3-benzodioxole as a colorless oil.

Isolation of 2,3-dihydroxybiphenyl. Cell extract [120 mg of protein], prepared from the parent strain of Beijerinckia; NAD^+, 10 µmoles; sodium pyruvate, 250 µmoles; lactic acid dehydrogenase, 2 mg of protein; in a final volume of 50 ml of 0.05M KH_2PO_4 buffer, pH 7.5 were placed in a stoppered serum bottle. The mixture was made anaerobic by flushing with nitrogen for 45 minutes. The reaction was started by the addition of 250 µmoles of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene in 2.0 mls of 0.05M KH_2PO_4 buffer, pH 7.5. After 1.0 hour the reaction was stopped by the addition of 5.0 mls

*Prepared according to Cookson et al. J. Chem. Soc., 1905 (1967).

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of 5N H_2SO_4. The precipitated protein was removed by centrifugation and the clear supernatant solution was extracted with ether. The ether extract was dried over anhydrous sodium sulfate and the solvent removed to give 42 mg of a brown oil. The residue was purified by vacuum sublimation and gave 17 mg of 2,3-dihydroxybiphenyl.

Analytical Methods. Ultraviolet and visible spectra were determined on a Cary model 14 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer. Crystalline samples were mulled in nujol and placed between NaCl discs. Noncrystalline samples were run on neat liquid films between NaCl discs. All absorptions were referenced to the absorptions of polystyrene. Parent ion molecular weights were determined by peak matching with assigned perfluoroalkane peak fragments. The determinations were made on a Dupont-Consolidated Electrodynamics Corporation Model 21-110 High Resolution Mass Spectrometer. Proton magnetic resonance [PMR] spectra were recorded on Varian A-60, HA-100 or Perkin-Elmer R-12 spectrometers. Absorptions were assigned δ values at the midpoint of half height and are referenced to tetramethylsilane [TMS]. The following abbreviations are used in PMR peak descriptions: s. [singlet], d. [doublet], t. [triplet], q. [quartet], m. [multiplet], and b. [broadened]. Melting points were obtained by use of a Büchi melting point apparatus and are uncorrected.

Materials. All chemicals were of the highest purity commercially available. Lactic acid dehydrogenase, sodium pyruvate and NAD^+ were from Sigma.

RESULTS

A bacterium, tentatively identified as a Beijerinckia species, was isolated from a polluted stream. Aromatic hydrocarbons that served as sole sources of carbon and energy for growth were biphenyl, naphthalene, anthracene, and phenanthrene. Benzene, toluene and ethylbenzene did not serve as growth substrates.

A mutant strain [Beijerinckia B8/36] of the above organism, that could

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no longer utilize biphenyl as a growth substrate, was obtained by treatment with N-methyl-N'-nitro-N-nitrosoguanidine. Beijerinckia B8/36, when grown on succinate in the presence of biphenyl, accumulated a neutral, ultraviolet-absorbing compound [I] in the culture medium. Ethyl acetate extraction of 10 liters of culture filtrate gave 4.60 g of a white solid which was recrystallized from hexane. The physical properties of compound I were as follows: mp 93 C; λ_{max}^{MeOH} 303 and 223 nm; ϵ_{303} = 13,600, ϵ_{223} = 9,200; λ_{max}^{nujol} 3.1 and 13.2 μ; calculated mass for $^{12}C_{12}{}^{1}H_{12}{}^{16}O_2$ 188.0831, found mass 188.0837. The PMR spectrum of compound I in $CDCl_3$ gave bands at 2.7 δ, 2H[b.s. hydroxyl]; 4.5 δ, 2H[d.d., hydroxymethine]; 5.9 δ, 2H[m, olefinic]; 6.35 δ, 1H[m, olefinic] and 7.4 δ, 5H[m, aromatic]. This data suggested that compound I was a phenyl-substituted, hydroxylated, cyclohexadiene derivative. This was supported by the acid-catalyzed dehydration of compound I to give 2-hydroxybiphenyl [66%] and 3-hydroxybiphenyl [34%]. Both phenols were isolated and shown to have identical melting points and infrared spectra to those given by synthetic samples. These observations show that compound I contained two adjacent hydroxyl groups. Final proof of the structure of compound I was obtained by cycloaddition of the diacetate of I with 4-[*p*-bromophenyl]-1,2,4-triazoline-3,5-dione to give 8,9-diacetoxy-1-phenyl-4-[*p*-bromophenyl]-2,4,6-triazatricyclo[5.2.2.$0^{2,6}$]undec-10-ene-3,5-dione [II]; mp 208-209 C; λ_{max}^{nujol} 5.65, 5.75, 5.82, 8.10 and 13.2 μ; calculated mass for $^{12}C_{24}{}^{1}H_{20}{}^{14}N_3{}^{16}O_6{}^{79}Br$, 525.0536, found mass 525.0539. Results of a 100 MHz spectrum of II are shown in Figure 1.

The above evidence establishes the structure of compound I, that is formed from biphenyl by Beijerinckia B8/36, as 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene. It should be noted that the results do not establish the cis-configuration of the hydroxyl groups. Preliminary evidence for this feature of the molecule was provided by the reaction of I with 2,2-dimethoxypropane to form [2]-3a,7a-dihydro-4-phenyl-2,2-dimethyl-1,3-benzodioxole. A 100 MHz PMR spectrum of this derivative in benzene-d_6 gave the results shown in Figure 2.

Crude cell extract, when incubated under anaerobic conditions with cis-

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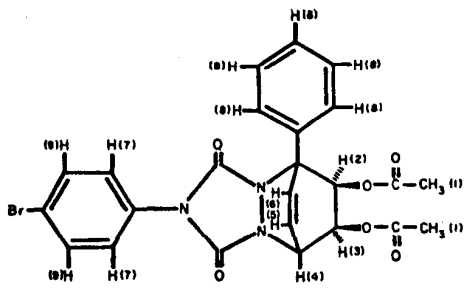

Proton	Chemical Shift δ	Description
1	1.70	3H [s, acetate methyl]
1	2.00	3H [s, acetate methyl]
2	5.90	1H [d, acetoxymethine, $J_{2,3}$ = 7.0 Hz]
3	5.80	1H [dd, acetoxymethine, $J_{3,2}$ = 7.0 Hz; $J_{3,4}$ = 4.0 Hz]
4	5.50	1H [m, bridgehead methine]
5	6.70	1H [dd, olefinic, $J_{5,6}$ = 8.0 Hz; $J_{5,4}$ = 5.0 Hz]
6	7.23	1H [d, olefinic, $J_{6,5}$ = 8.0 Hz]
7	7.32	2H [m, aromatic ortho to nitrogen]
8	7.50	5H [s, aromatic]
9	7.87	2H [dd, aromatic ortho to bromine]

FIGURE 1: Analysis of the Proton Magnetic Resonance Spectrum of 8,9-Diacetoxy-1-phenyl-4-[p-bromophenyl]-2,4,6-triazatricyclo[5.2.2.$0^{2,6}$] undec-10-ene-3,5-dione.

The sample was dissolved in deuterated pyridine and the spectrum recorded at 100 MHz. Tetramethylsilane was used as an internal standard.

2,3-dihydroxy-1-phenylcyclohexa-4,6-diene led to the formation of 2,3-dihydroxybiphenyl. The latter compound was isolated and purified by sublimation. Its properties mp 110 C; λ_{max}^{MeOH} 391 and 247 nm, ϵ_{391} = 2,270, ϵ_{247} = 9,840; λ_{max}^{nujol} 2.8, 3.0, 6.20, 6.32, 6.40, 7.30, 13.3, and 14.3 μ; calculated mass for $^{12}C_{12}{}^{1}H_{10}{}^{16}O_2$, 186.0681, found mass 186.0679, were consistent with

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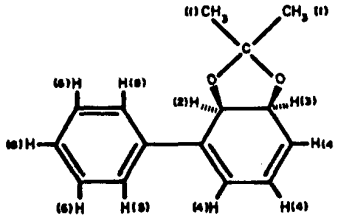

Proton	Chemical Shift δ	Description
1	1.33	3H [s, methyl]
1	1.35	3H [s, methyl]
2	4.75	1H [d, alkoxymethine, $J_{2,3}$ = 8.0 Hz]
3	4.62	1H [dd, alkoxymethine, $J_{3,2}$ = 8.0 Hz; $J_{3,4}$ = 1.0 Hz]
4	5.77	2H [m, olefinic]
4	6.15	1H [m, olefinic]
5	7.60	2H [m, aromatic]
6	7.15	3H [m, aromatic]

FIGURE 2: Analysis of the Proton Magnetic Resonance Spectrum of [Z]-3a,7a-Dihydro-4-phenyl-2,2-dimethyl-1,3-benzodioxole.

The sample was dissolved in deuterated benzene and the spectrum recorded at 100 MHz. Tetramethylsilane was used as an internal standard.

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the proposed structure. Further support was provided by its PMR spectrum in $CDCl_3$ which showed bands at 5.2 δ, 2H[b.s. hydroxyl]; 6.8 δ, 3H and 7.45 δ, 5H [aromatic protons].

The initial reactions utilized by Beijerinckia to oxidize biphenyl are shown in Figure 3.

DISCUSSION

It appears that mammals oxidize aromatic hydrocarbons to arene oxides which yield trans-arenediols by enzymatic hydration (6). Until recently it

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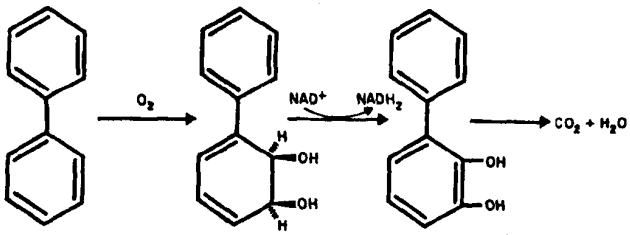

FIGURE 3: Initial Reactions in the Oxidation of Biphenyl by Beijerinckia.

was assumed that bacteria also oxidize aromatic hydrocarbons through trans-arenediols (7). However, P. putida incorporates two atoms of molecular oxygen into benzene with the formation of cis-1,2-dihydroxycyclohexa-3,5-diene (8). cis-Arenediols have also been identified as intermediates in the bacterial oxidation of toluene (9), p-chlorotoluene (10), ethylbenzene (11), naphthalene (12,13), and benzoic acid (14). In addition, unpublished work in our laboratory has shown that cis-arenediols are also formed from p-fluorotoluene, p-bromotoluene and chlorobenzene. The identification of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene as an intermediate in the oxidation of biphenyl suggests that cis-hydroxylation is a common reaction in the bacterial degradation of aromatic hydrocarbons (15). However, it should be noted that cis-stereochemistry of the biphenyl metabolite is inferred by its ability to form an isopropylidine derivative with 2,2-dimethoxypropane (16). The absolute stereochemistry of cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene is presently being determined by X-ray analysis of the Diels-Alder adduct.

The identification of 2,3-dihydroxybiphenyl as a further intermediate in biphenyl metabolism confirms the observations of Lunt and Evans (2). These authors also reported that 2,3-dihydroxybiphenyl is enzymatically oxidized to α-hydroxy-δ-phenylmuconic semialdehyde. Cell extracts prepared from the wild type strain of Beijerinckia oxidized 2,3-dihydroxybiphenyl to a yellow

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product whose spectral characteristics in acid and alkaline solution were identical to those reported for α-hydroxy-β-phenylmuconic semialdehyde.

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