Aromatization of a Bicyclic Steroid Analog, 3-Oxodecalin-4-ene-10-carboxaldehyde, by Liver Microsomal Cytochrome P450 2B4†

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ABSTRACT: Several purified isoforms of microsomal cytochrome P450 were previously shown in this laboratory to catalyze the oxidative deformylation of a variety of α- or β-branched aldehydes with the production of olefins and formic acid. In the present study, 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC, numbered according to the convention for steroids) was synthesized as a bicyclic analog of the aldehyde that is known to be the terminal intermediate in the enzymatic conversion of androgens to estrogens. ODEC undergoes aromatization in a reconstituted enzyme system containing liver microsomal cytochrome P450 2B4 and NADPH-cytochrome P450 reductase, along with NADPH and phosphatidylcholine, under aerobic conditions. The products, 3-hydroxy-6,7,8,9-tetrahydronaphthalene (HTN) and formic acid, were identified by mass spectrometry. The corresponding 10-carbinol does not undergo oxidative aromatization with P450 2B4, and with ODEC as substrate, other microsomal P450 cytochromes are either weakly active (isoforms 2C3 and 3A6) or inactive (isoforms 2E1, 1A2, and 2G1). Cytochrome b_5 stimulates the P450 2B4-catalyzed reaction with ODEC about 2.6-fold but has no effect with the other P450s. In two respects the conversion of the bicyclic model compound to HTN with P450 2B4 was shown to be similar to that of the steroid aromatase reaction. Deuterium in the formyl group of ODEC was retained in the formic acid that was produced and isolated as the 4-nitrobenzyl derivative, and with preparations of ODEC containing deuterium in the 1α position or the 1α and 2α positions, it was shown that the desaturation reaction is specific for removal of the 1β-hydrogen, thus involving a stereospecific *cis* elimination of formate. Cytochrome b_5 has no effect on the stereospecificity of the reaction.

The role of estrogen hormones in normal endocrine function (Witiak et al., 1989) and estrogen-dependent disorders such as endometrial and breast cancer (Johnston & Metcalf, 1984; Brodie, 1985; Santen et al., 1987) and benign prostatic hyperplasia (Henderson et al., 1987) has stimulated investigations on aromatase, the P450[1] enzyme that catalyzes the NADPH- and O_2-dependent conversion of androgens to estrogens (Meyers, 1955b; Thompson & Siiteri, 1974; Kellis & Vickery, 1987). From an enzymological viewpoint, the aromatase is of much interest because of the unusual mechanism by which, in three sequential oxidative reactions, it effects demethylation of the 10β-methyl group on the androgen nucleus (Cole & Robinson, 1990a). As shown in Figure 1A, the 10β-methyl carbon is oxygenated to yield the 10β-hydroxymethyl compound and then the 10β-formyl compound as transient intermediates (Meyer, 1955a,b; Morato et al., 1961; Cole & Robinson, 1990a). In the terminal reaction, oxidative deformylation occurs to yield the estrogen and formic acid (Skinner & Akhtar, 1969). The mechanism of this unique cleavage reaction is unclear and has been the subject of prolonged discussion (Morato et al., 1962; Morand et al., 1975; Mastalerz & Morand, 1982; Caspi et al., 1984; Caspi & Njar, 1987; Covey et al., 1984, 1987; Hahn & Fishman, 1984; Fishman & Hahn, 1987; Akhtar et al., 1982; Stevenson et al., 1988; Watanabe & Ishimura, 1989; Korzekwa et al., 1991). Some details of the terminal reaction are known; for example, the formate that is generated contains the aldehydic hydrogen of the 10β-formylandrogen intermediate as well as an atom of oxygen

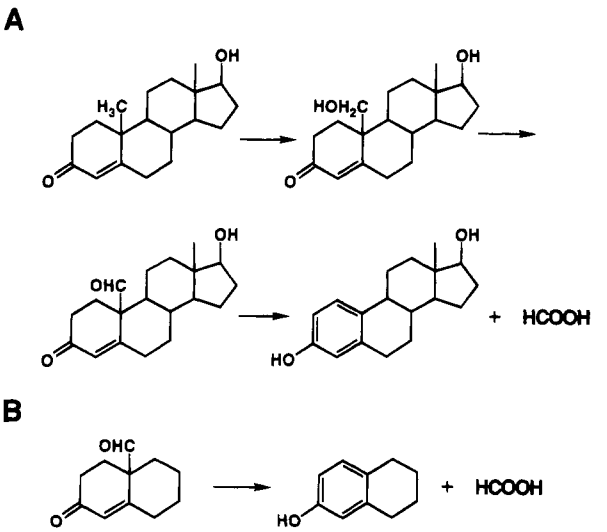

FIGURE 1: A, Conversion of testosterone to estradiol, catalyzed by steroid aromatase; B, conversion of ODEC to HTN, catalyzed by P450 2B4.

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[1] Abbreviations: P450, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroylglyceryl-3-phosphorylcholine; SOD, superoxide dismutase; ODEC, 3-oxodecalin-4-ene-10-carboxaldehyde; HTN, 3-hydroxy-6,7,8,9-tetrahydronaphthalene; NOE, nuclear Overhauser effect; COSY, 2-dimensional homonuclear correlation spectroscopy, GC/MS-EI, coupled gas chromatography−mass spectroscopy with electron impact ionization. To indicate the similarity of the aromatization reaction to that of testosterone, the rings of ODEC and HTN are numbered according to the convention for the A and B rings of steroids.

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derived from O_2 (Akhtar et al., 1982). The 1β-hydrogen of testosterone or androstenedione is stereospecifically removed, appearing in water (Morato et al., 1962; Talalay, 1965; Townsley & Brodie, 1968; Fishman et al., 1969), whereas only with androstenedione is removal of the 2β-hydrogen stereospecific (Fronckowiak & Osawa, 1988; Cole & Robinson, 1990b).

We have previously described the oxidative deformylation of xenobiotic aldehydes such as cyclohexanecarboxaldehyde and citronellal to olefins and formate by purified liver microsomal P450 enzymes (Vaz et al., 1991; Roberts et al., 1991) and have suggested this reaction as a possible model to elucidate the mechanism of the terminal step in steroid aromatization. To this end, as briefly reported elsewhere (Vaz et al., 1992), we have demonstrated the P450 2B4-dependent aromatization of an aldehyde, 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC), that is an analog of the A and B rings of the 10β-formylandrogen intermediate, as shown in Figure 1B. We describe herein the chemical synthesis of this analog and its specifically deuterated counterparts and some key aspects of the aromatization by purified liver microsomal P450 2B4 in a reconstituted enzyme system, including the stereochemistry of hydrogen atom removal. The aromatization of ODEC by P450 2B4 has features common to the terminal step of steroid aromatization, which leads us to propose the oxidative deformylation of ODEC by liver microsomal P450 as a particularly relevant mechanistic model for the terminal step in the conversion of estrogens to androgens.

MATERIALS AND METHODS

Chemical Synthesis of ODEC. All reagents used in the synthetic procedures were obtained commercially, and the source is indicated for each compound used. Melting and boiling points are uncorrected. IR spectra of compounds in potassium bromide films were recorded with a Nicolet DX-5 FTIR spectrometer. The most characteristic signals are reported in wavenumbers, and the functional group associated with the wavenumber is identified. For silica gel column chromatography, a Michel–Miller column (40 × 350 mm; Ace Glass, Vineland, NJ) packed with 32- to 63-μm particle size silica gel (Selecto Inc., Kennesaw, GA) was used and all elutions were done stepwise. The procedure for synthesizing ODEC, a compound that has not been described previously, is given below and summarized in Figure 2A. The intermediates and the final product were all the racemic compounds.

A Michael condensation (step a) was used to form the decalin ring system appropriately functionalized at positions 3, 4, and 10. To 19 g of ethyl 2-oxo-cyclohexanecarboxylate (Aldrich, Milwaukee, WI) and 1.0 g of sodium *tert*-butoxide (Aldrich) in 20 mL of anhydrous ethanol at room temperature was added a mixture of 11 mL of methyl vinyl ketone (Aldrich) and 10 mL of anhydrous ethanol dropwise with stirring over a period of 2 h. The reaction mixture was heated under reflux for 1 h and then cooled to room temperature, and 3 mL of concentrated sulfuric acid was added dropwise with stirring. The mixture was stirred overnight (16 h) at room temperature and then diluted with 200 mL of water and extracted with 200 mL of chloroform. The chloroform layer was washed with saturated sodium bicarbonate and dried over anhydrous sodium sulfate, and

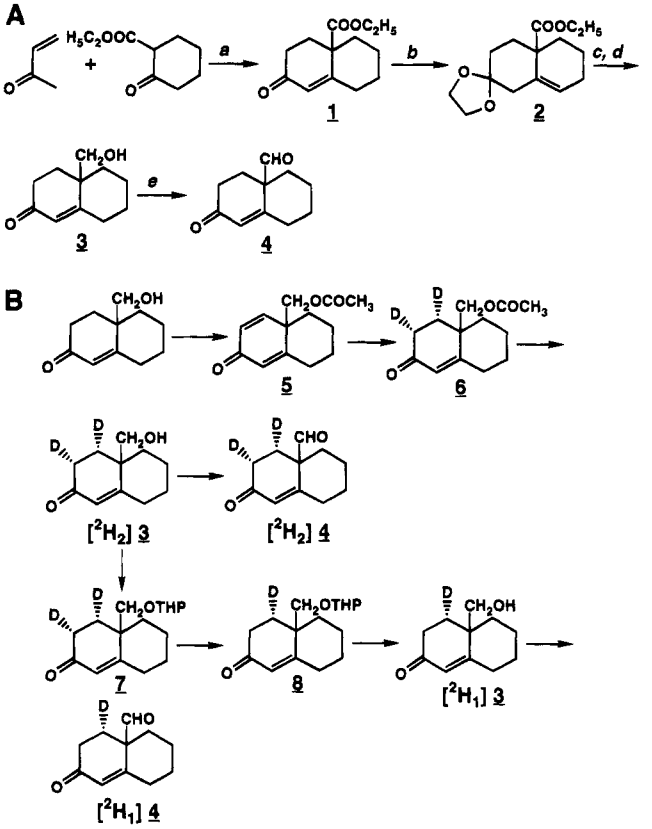

FIGURE 2: A, Procedure for chemical synthesis of ODEC (compound **4**); B, synthesis of ODEC with deuterium at positions 1 and 2 (compound [$^2H_2$]**4**) or at position 1 only (compound [$^2H_1$]**4**).

the solvent was removed under reduced pressure. Vacuum distillation of the residue gave 22 g (88% yield) of compound **1**, ethyl 3-oxodecalin-4-ene-10-carboxylate (bp 146 °C at 3 mmHg), greater than 95% pure as judged by GC/MS. This intermediate was used without further purification: IR (KBr) 1722 cm^{-1} for the $COOC_2H_5$ group and 1679 cm^{-1} for the C=C–C=O group; GC/MS-EI *m/z* 222 (M^+, 24), 194 (M^+ − CO, 5.0), 166 (M^+ − CH_2CH_2CO, 9.8), 149 (M^+ − $COOC_2H_5$, 100), 148 (M^+ − $COOC_2H_5$ − H, 79), 138 (M^+ − CO − CH_2CH_2CO, 30), 107 (M^+ − $COOC_2H_5$ − CH_2-CO, 82).

Compound **1** (21 g, obtained as above) in 200 mL of dry benzene containing 0.5 g of *p*-toluenesulfonic acid and 25 mL of ethylene glycol was heated under reflux for 6 h with azeotropic removal of water. The benzene solution was washed with saturated sodium bicarbonate and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. Vacuum distillation of the residue yielded 18 g (71% yield) of compound **2**, ethyl 3,3-ethylenedioxydecalin-5-ene-10-carboxylate: bp range 160–165 °C at 3 mmHg; IR (KBr) 1722 cm^{-1} for the $CO_2C_2H_5$ group; GC/MS-EI *m/z* 266 (M^+, 28), 238 (M^+ − CO, 1), 193 (M^+ − $COOC_2H_5$, 6), 99 (M^+ − $COOC_2H_5$ − C_7H_{10}, 100).

The ethylene ketal (21 g) in 100 mL of anhydrous diethyl ether was added dropwise with stirring to 3 g of lithium aluminum hydride in 300 mL of anhydrous ether. The reaction mixture was then heated under reflux for 6 h, and the excess lithium aluminum hydride was destroyed by the dropwise addition of dry ethanol (20 mL) followed by 200 mL of cold 1 M sulfuric acid. The reaction mixture was stirred for 2 h, following which the ether layer was separated

and the solvent was removed under reduced pressure to yield 3,3-ethylenedioxydecalin-5-ene-10-carbinol as an oil. This intermediate was hydrolyzed at room temperature by stirring overnight (16 h) in 1 M sulfuric acid (200 mL in 25% aqueous ethanol). The reaction mixture was concentrated under reduced pressure to approximately 100 mL and then continuously extracted with 200 mL of ether. The ether extract was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to yield an oil, which was dissolved in 50 mL of chloroform and submitted to chromatography on a silica gel column. The column was successively treated with chloroform−acetone, 90:10 (250 mL), 80:20 (250 mL), and 70:30 (500 mL), the last of which removed the product. Fractions containing the product were pooled and evaporated under reduced pressure to give 10 g (70% yield) of compound **3**, 3-oxodecalin-4-ene-10-carbinol: mp, 77−79 °C; IR (KBr) 3416 cm^{-1} for the CH_2OH group and 1651 cm^{-1} for the C=C−C=O group; GC/MS-EI *m/z* 180 (M^+, 24), 150 ($M^+ - CH_2O$, 100), 149 ($M^+ - CH_2OH$, 33), 122 ($M^+ - CH_2O - C_2H_4$, 33), 107 ($M^+ - CH_2OH - CH_2CO$, 56); NMR (500 MHz, deuteriochloroform) δ 5.8 (1H), 3.7 (doublet, 2H), 2.6 (multiplet, 1H), 2.3 (multiplet, 3H), 2.2 (multiplet, 1H), 2.0 (multiplet, 1H), 1.9 (multiplet, 1H), 1.6 (multiplet, 3H), 1.4 (multiplet, 1H), 1.2 (multiplet, 1H).

For mild oxidation of the 10-hydroxymethyl compound, 1 g dissolved in 50 mL of anhydrous methylene chloride was added at room temperature with vigorous stirring to a finely ground suspension of 1.7 g of pyridinium chlorochromate (Aldrich) in 200 mL of anhydrous methylene chloride. After 1 h, 250 mL of anhydrous diethyl ether was added while stirring was maintained. The reaction mixture was decanted and filtered through a bed of neutral alumina, the bed was washed with 100 mL of anhydrous ether, and the combined solution was concentrated under reduced pressure at room temperature. The residue was dissolved in 10 mL of chloroform and applied to a silica gel column (25 × 290 mm). The column was treated with 250 mL of chloroform, and fractions containing the product were pooled and concentrated under reduced pressure at room temperature to give 0.7 g (71% yield) of the desired ODEC (compound **4**) as a low-melting solid: mp > −20 and <0 °C; IR (KBr) 1722 cm^{-1} for the CHO group and 1672 cm^{-1} for the C=C−C=O group; GC/MS-EI *m/z* 178 (M^+, 28), 160 ($M^+ - H_2O$, 2), 150 ($M^+ - CO$, 28), 149 ($M^+ - HCO$, 80), 121 ($M^+ - HCO - CO$, 35), 107 ($M^+ - HCO - CH_2CO$, 100), 93 ($M^+ - HCO - CH_2CH_2CO$, 40).

Deuterium-Labeled ODEC. 3-Oxodecalin-4-ene-10-[$^2H_2$]-carbinol was synthesized from compound **2** by the method already used for the synthesis of compound **3**, except that in step c $LiAlD_4$ of >98% isotopic purity (Aldrich) was the reductant. The oxidation of dideuterio **3** to monodeuterio **4**, 3-oxodecalin-4-ene-10-[^{2}H]carboxaldehyde, was as in step e.

The procedures used for stereospecific deuteration of ODEC in the ring are shown in Figure 2B. Prior to deuteration at the 1α and 2α positions, compound **3** was acetylated and then dehydrogenated to give 3-oxodecalin-1,4-diene-10-methyl acetate, **5**. In this procedure, a solution of compound **3** (1.8 g) in 2.0 mL of acetic anhydride and 10 mL of acetic acid was warmed at 60 °C for 3 h, following which 5.7 g of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (Aldrich) in 150 mL of dry benzene was added. The mixture was heated under reflux for 18 h, after which the solvent was removed under reduced pressure and the residue was taken up in 50 mL of anhydrous chloroform. After removal of the insoluble residue by filtration through a glass wool plug, the chloroform solution was applied to a column having a 150-mL bed volume of neutral alumina (Aldrich). The column was treated with 200 mL of 30% acetone in chloroform, and the eluate was concentrated under reduced pressure to yield an oil, which was dissolved in 20 mL of chloroform and submitted to chromatography on a silica gel column (40 × 350 mm). The column was treated with 250 mL of chloroform containing 5% acetone and then with 500 mL of chloroform containing 20% acetone, which eluted the product. Fractions containing compound **5** were pooled and concentrated under reduced pressure to yield 1.1 g (60% yield) of product that crystallized on standing overnight: mp 57−60 °C; IR (KBr) 1744 cm^{-1} for the acetate group and 1666 cm^{-1} for the $(C=C)_2C=O$ group; GC/MS-EI 220 *m/z* (M^+, 2), 148 ($M^+ - CHOCOCH_3$, 91), 120 ($M^+ - CHOCOCH_3 - CH_2CH_2$, 48), 43 ($CH_3CO$, 100).

The reduction of **5** to [1α,2α-$^2H_2$]-3-oxodecalin-4-ene-10-carbinol was done by the stereospecific method of Djerassi and Gutzwiller (1966). In a glass pressure reaction vessel fitted with a Teflon adapter (Ace Glass) to 115 mg of norbornadienerhodium(I) chloride (Chemical Dynamics, South Plainfield, NJ) dissolved in 85 mL of anhydrous benzene were added 400 mg of triphenylphosphine (Aldrich) in 80 mL of 95% ethanol and then 1.0 g of **5** in 5 mL of 95% ethanol. The vessel was sealed and evacuated with a vacuum pump, D_2 gas of 98% isotopic purity (Aldrich) was introduced, and the pressure was maintained at 20 psi. The reaction mixture was stirred vigorously for 58 h at room temperature, after which the solvent was removed under reduced pressure and the residue (compound **6**) was dissolved in 50 mL of 95% ethanol, and 4.5 mL of 36% aqueous HCl was added. The solution was stirred overnight (16 h) at room temperature, and the solvent was removed under reduced pressure. The residue was dissolved in 20 mL of chloroform and applied to a column with a 50-mL bed volume of silica gel, and elution was carried out with 200 mL of 1:1 chloroform−acetone. The eluate was evaporated under reduced pressure, and the residue was dissolved in 25 mL of chloroform and submitted to chromatography on a silica gel column (40 × 350 mm). Elution was carried out as described above for unlabeled **3** with chloroform containing increasing amounts of acetone, and the fractions containing the product were pooled and evaporated under reduced pressure to yield 0.9 g (88% yield) of deuterated compound **3**: mp 78−81 °C; IR (KBr) 3414 cm^{-1} for CH_2OH and 1649 cm^{-1} for the C=C=O group. The NMR spectra are given in Figure 3 and discussed below. The oxidation of this intermediate (0.1 g) to a preparation containing [1α,2α-$^2H_2$]-ODEC, dideuterio compound **4**, was as described above. As indicated below, the preparation also contained some monodeuterated and nondeuterated ODEC.

Monodeuterio compound **4**, [1α-$^2H_1$]ODEC, was obtained from dideuterio **3** after protection of the hydroxymethyl function as the tetrahydropyranyl (THP) ether **7**. To 200 mg of [$^2H_2$]**3** dissolved in 10 mL of chloroform were added 150 μL of dihydropyran (Aldrich) and 5.0 mg of *p*-toluenesulfonic acid. The mixture was stirred at room temperature for 2 h, and the chloroform solution was then applied to a column having a 10-mL bed volume of basic

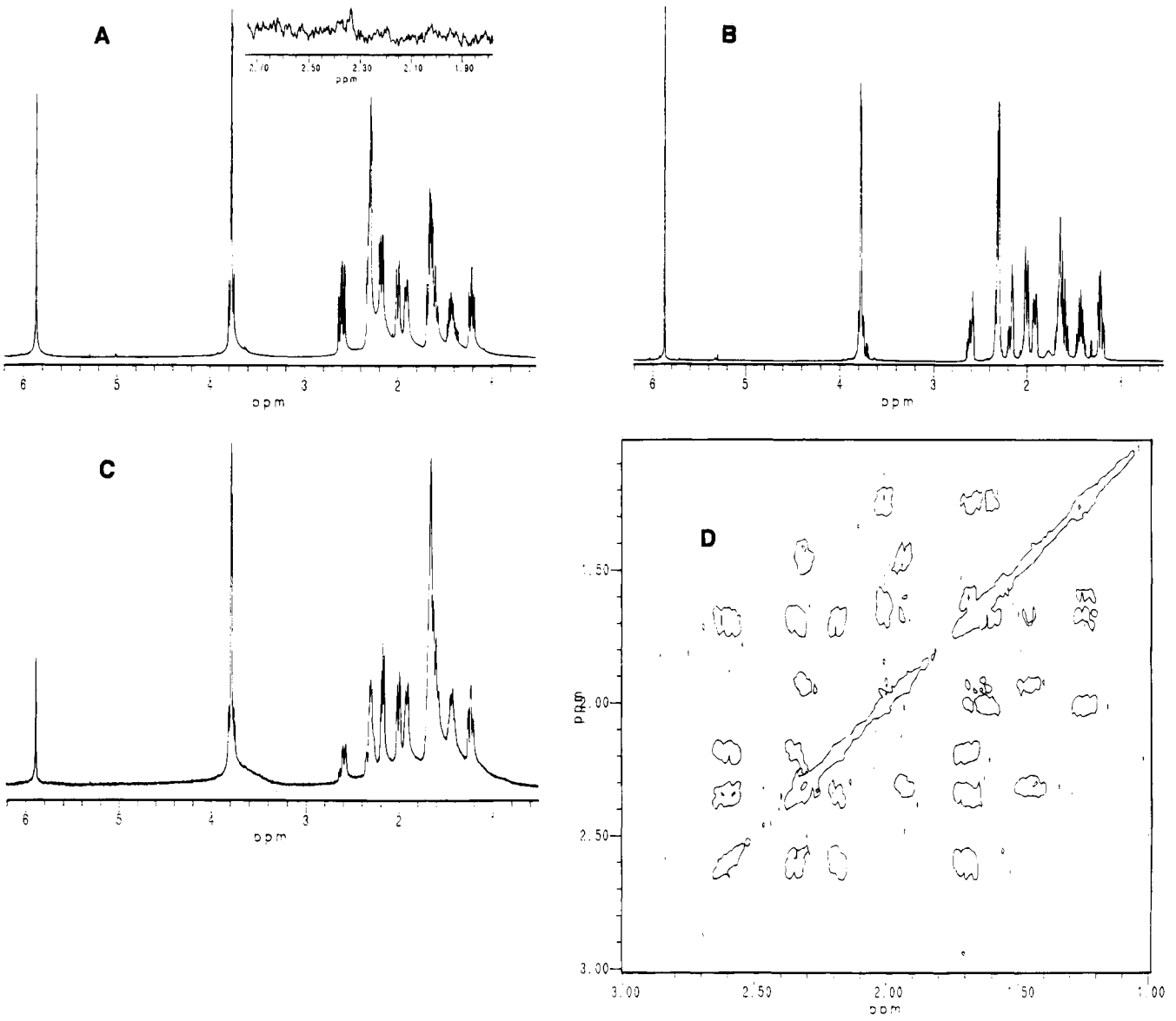

FIGURE 3: 500-MHz proton NMR spectra of 3-oxodecalin-4-ene-10-carbinol and deuterio isotopomers. Panel A, 3-oxodecalin-4-ene-10-carbinol with the inset showing the NOE effect due to irradiation of the proton signals at 3.8 ppm. Panel B, [1α,2α-$^2H_2$]-3-oxodecalin-4-ene-10-carbinol synthesized as described in the text. Panel C, [1,4,6-$^2H_5$]-3-oxodecalin-4-ene-10-carbinol synthesized by base-catalyzed exchange of enolizable hydrogens of the THP ether of compound **3** in 1:1 ethanol-OD−D_2O followed by acid hydrolysis in 1:1 ethanol-OD−D_2O. Panel D, 2-D homonuclear COSY spectrum of 3-oxodecalin-4-ene-10-carbinol.

alumina (Aldrich), which was washed with 50 mL of chloroform containing 10% acetone. The eluate was concentrated under reduced pressure to give compound **7**, the THP ether, as an oil, which was dissolved in 15 mL of anhydrous ethanol containing 0.5 N sodium *tert*-butoxide. The solution was allowed to stand at room temperature for 24 h to give compound **8**, after which 15 mL of 5% aqueous HCl were added and the solution was stirred for 8 h at room temperature to hydrolyze the ether. The reaction mixture was then extracted with 50 mL of chloroform, and the extract was dried over anhydrous sodium sulfate, concentrated to approximately 10 mL under reduced pressure, and submitted to chromatography on a silica gel column as described above for unlabeled **3**. Compound [$^2H_1$]**3** was subsequently oxidized to [1α-$^2H_1$]ODEC as described above. We present evidence below that the preparation contained the unlabeled form and a small amount of the 2α-monodeuterio compound as well.

Enzyme System for ODEC Aromatization. The P450 cytochromes and the reductase were purified from rabbit liver microsomes by methods summarized elsewhere (Koop et al., 1982; Morgan et al., 1983; Ding & Coon, 1992; French & Coon, 1979). Rabbit liver microsomal cytochrome b_5 was purified by the method of Strittmatter et al. (1978), and catalase (bovine liver, thymol-free), SOD (bovine erythrocyte), glucose 6-phosphate dehydrogenase (type V), and glucose oxidase (type II-S) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine milk xanthine oxidase was a gift from Dr. Vincent Massey of this department.

For reconstitution of the P450 enzyme system, appropriate volumes of stock solutions of P450, reductase, and cytochrome b_5 (when included) were mixed at the desired concentrations and a suitable volume of a freshly sonicated aqueous dispersion of DLPC (1.0 mg/mL) was added so that the phospholipid concentration in the final reaction mixture

was 30 μg/mL. The reconstituted enzyme system was allowed to stand on ice for 20−60 min prior to use. The complete system included, in addition to these components, 50 mmol of potassium phosphate buffer, pH 7.4, 1.0 μmol of ODEC (from a 0.1 M stock solution in 95% ethanol), and 2.0 μmol of NADPH as the last addition in a final 1.0-mL reaction volume. The assays were at 37 °C for 20 min.

Quantitative Analysis of HTN. In assays of ODEC aromatization, after incubation of the reaction mixture for the appropriate time, the reaction was quenched by the addition of 2.0 mL of methylene chloride with vigorous mixing and 60 μL of a 0.1 mM solution of 4-*tert*-butylphenol in 95% ethanol was added with mixing followed by centrifugation for 5 min to separate the layers. *tert*-Butylphenol is a useful internal standard with properties similar to those of HTN, including solubility, absorption maximum, and molar absorption coefficient. The methylene chloride layer was removed and taken to dryness in a vacuum centrifuge, and the residue was dissolved in 0.4 mL of 1:3 methanol−water. An aliquot of this solution (50−100 μL) was injected onto a NovaPak C-18 reversed phase column (5.0-μm particle size; Millipore Waters Chromatography Division, Millford, MA) fitted to a Waters HPLC system consisting of a model 600 multisolvent delivery system, a model 490 programmable detector, and a model 710 WISP autosampler. A model 3390A Hewlett-Packard recording integrator was used to determine peak intensities. An isocratic solvent system consisting of 1:1 methanol and 0.1% aqueous trifluoroacetic acid was used at a flow rate of 1.0 mL/min. The detector response at 282 nm, as determined by the integrated peak area, was linear between 100 and 2000 pmol. The amount of product formed was corrected for recovery on the basis of the internal standard. Figure 4 shows a typical HPLC analysis used for quantitation of HTN, where this product appeared at 11.1 min and the internal standard, *tert*-butylphenol, at 15.7 min.

Mass Spectral Analyses. GC/MS was performed with an S&W 30-m DB-5 fused silica capillary column fitted on a Finigan 40-21GC-MSDS mass spectrometer, under electron impact conditions at 70 eV. In the standard procedure, a sample in 2−5 μL of methylene chloride was injected onto the column, which was held for 2 min at 50 °C, followed by an increase to 275 °C at 20 °C/min. The column was held at the latter temperature for 30 min. The injector temperature was 200 °C, and the carrier gas was helium at a head pressure of 10 psi.

RESULTS

Structure Analysis of [1α,2α-$^2H_2$]-3-Oxodecalin-4-ene-10-carbinol. The methodology used to obtain 1α,2α-dideuteration of the bicyclic ring system is similar to that utilized by Djerassi and Gutzwiller (1966) for 1α,2α-dideuteration of steroids. The complete assignments of hydrogen resonances of 3-oxodecalin-4-ene-10-carbinol have been done by a combination of deuterium exchange, proton decoupling, COSY connectivity, and NOE signal enhancement experiments on the 500-MHz proton NMR spectrum. Figure 3, panel A, shows the proton NMR of 3-oxodecalin-4-ene-10-carbinol in the region from 1.0 to 6.0 ppm. The singlet at 5.8 ppm corresponds to the C_4 vinyl hydrogen. The doublet at 3.8 ppm is reduced to a singlet after repeated exchange of the hydroxyl hydrogen (in ethanol-OD, containing 0.1% trifluoroacetic acid, data not shown), consistent with an assignment of this resonance signal to the hydroxymethylene hydrogens. The other proton signals are observed between 2.7 and 1.1 ppm. Deuteration of compound **5** (Figure 2) by the method of Djerassi and Gutzwiller (1966) to yield [1,2-$^2H_2$]-3-oxodecalin-4-ene-10-carbinol results in the single proton signals at 2.6 and 2.2 ppm (Figure 3, panel B) being decreased in intensity, indicating that signals at these positions are due to hydrogens at the C_1 and C_2 positions. Additionally, base-catalyzed deuterium exchange of the THP ether of compound **3** (triethylamine−ethanol-OD−D_2O, 0.2:1.0:1.0 v/v; 60 °C, 24 h) followed by proton NMR analysis (Figure 3, panel C) of the resultant compound **3** (after acid-catalyzed removal of the THP group) indicated loss of signal intensity at 5.8, 2.6, and 2.3 ppm; this is consistent with the assignment of these signals to hydrogens at the C_4, C_2, and C_6 carbons, respectively. In proton decoupling experiments only resonances with integrations for a single hydrogen were examined. Decoupling the resonance signal at 2.6 resulted in pertubation of the multiplets at 2.3 and 1.6 ppm and collapse of the multiplet at 2.2 ppm to a doublet of doublets; similarly decoupling the resonance at 2.2 ppm results in pertubation of the multiplets at 2.3 and 1.6 ppm and collapse of the multiplet at 2.6 ppm to a doublet of doublets. All other resonance signals remained unaffected. Decoupling the resonance at 1.9 and 2.0 ppm resulted in pertubation of the multiplets at 1.6 and 1.4 ppm but had no effect on resonances at 2.6, 2.3, and 2.2 ppm. These results taken in conjunction with the deshielding effect of the keto group at C_3 and the double bond at C_4 are consistent with the assignment of the resonances between 2.6 and 2.2 ppm to hydrogens at C_2, C_6, and C_1 of the bicyclic ring system. Irradiation of the hydroxymethyl signal at 3.8 ppm results in a weak NOE enhancement of the signal at 2.3 ppm, whereas the signals at 2.6 and 2.2 ppm remain unaffected (Figure 3, panel A, inset). This indicates that through-space spin−spin interaction with the hydroxymethylene hydrogens is with the $C_{2\beta}$ or C_6 protons. With use of the assignments of the C_4 vinyl and C_{10} hydroxymethylene hydrogens, the resonances for the other hydrogens were also established from the proton COSY spectrum shown in Figure 3, panel D. These experiments have resulted in assignment of the proton resonances as follows: $H_{1\alpha}$, 2.2 ppm (1H); $H_{1\beta}$, $H_{7\beta}$, $H_{8\beta}$, 1.6 ppm (3H); $H_{2\alpha}$, 2.6 ppm (1H); $H_{2\beta}$, $H_{6\alpha}$, $H_{6\beta}$, 2.3

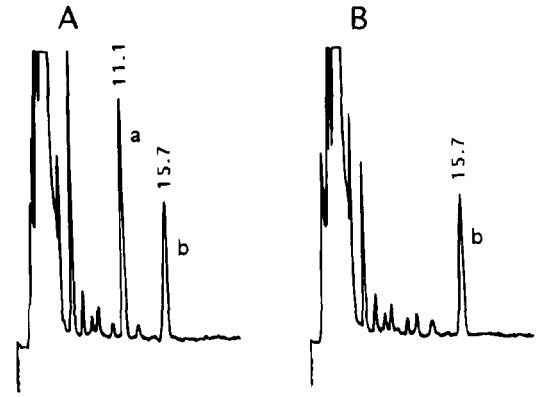

FIGURE 4: HPLC analysis of HTN as product of ODEC aromatization. The enzyme assay was carried out as described in Materials and Methods, with 4-*tert*-butylphenol as an internal standard. The profiles are shown for, A, the complete reaction mixture and, B, with the P450 and reductase heat-inactivated, where a is the HTN peak and b is the 4-*tert*-butylphenol peak.

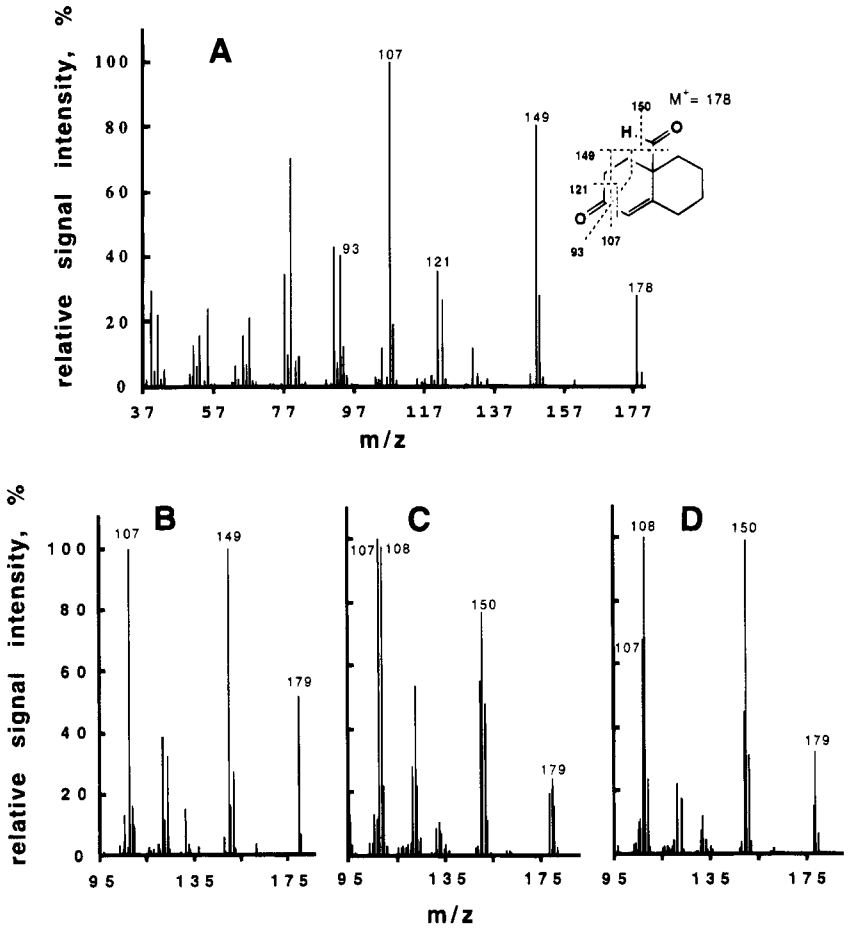

FIGURE 5: Mass spectra of ODEC (A) and the three deuterated forms of ODEC, [^{2}H]formyl (B), 1α,2α$^2H_2$ (C), and 1α$^2H_1$ (D), with mass per unit charge (*m/z*) shown as a function of ion intensity. The fragmentation pattern for ODEC is given in the inset to panel A.

ppm (3H); $H_{9\beta}$, 2.0 ppm (1H); $H_{8\alpha}$, 1.9 ppm (1H); $H_{9\alpha}$, 1.2 ppm (1H); $H_{7\alpha}$, 1.4 ppm (1H). The results from these NMR experiments establish that the method of Djerassi and Gutzwiller (1966) used for the deuteration of ODEC at the C_1 and C_2 positions results in stereospecific deuteration of the α face of the bicyclic ring system.

Determination of Isotope Distribution in Deuterium-Labeled ODEC. The mass spectra of chemically synthesized ODEC and the three deuterated forms, formyl-^{2}H, 1α,2α-$^2H_2$, and 1α-$^2H_1$, are shown in Figure 5, and the fragmentation pattern of the undeuterated form is given in the inset of panel A. The deuterium content at the aldehyde carbon of [*formyl*-^{2}H]ODEC was greater than 98% as determined from the signal intensities at *m/z* 178 and 179 (panel B). The fragmentation pattern established for unlabeled ODEC provides unique ion masses at *m/z* 107 and 108 that can be used to determine the deuterium content at the C_1 position in the two ring-labeled compounds, 1α-$^2H_1$ and 1α,2α-$^2H_2$. In unlabeled ODEC, the intensity of the *m/z* 108 ion mass is 19% of that at *m/z* 107 and the *m/z* 179 ion intensity is 14% of that at *m/z* 178. Accordingly, the *m/z* 108, 179, and 180 ion mass intensities in panels C and D were corrected by subtracting 19% of the corresponding ion mass intensity at *m/z* 107 and 14% of the corresponding ion mass intensities at *m/z* 178 (for *m/z* 179) and *m/z* 179 (for *m/z* 180), respectively. From the corrected *m/z* 108 ion mass intensities, the deuterium content at the C_1 position was established to be 44%. No single ion mass in the fragmentation pattern of ODEC exclusively identified the C_2 position. Accordingly, the deuterium content at C_2 was deduced from the corrected signal intensities at *m/z* 108, 179, and 180 as follows. The intensities at *m/z* 178, 179, and 180 indicate that [1α,2α-$^2H_2$]**4** was an isotopic mixture of undeuterated (*m/z* 178, 36.3%), 1α,2α-$^2H_2$ (*m/z* 180, 23.4%), and 1α-$^2H_1$ or 2α-$^2H_1$ (*m/z* 179, 40.3%) substrate. The corrected ion current intensity at *m/z* 108 indicates the deuterium content at C_1 as the sum of the 1α-$^2H_1$ and 1α,2α-$^2H_2$ components in the mixture (44%), whereas the corrected *m/z* 180 ion current intensity is due to the 1α,2α-$^2H_2$ component alone (23.4%). Accordingly, the 1α-$^2H_1$ component is established to be 20.6% of the mixture. The corrected *m/z* 179 ion intensity (40%) is due to the 1α-$^2H_1$ and 2α-$^2H_1$ components in the mixture; hence, the 2α-$^2H_1$ component is 19.4% of the isotopic mixture. Table 1 shows the deuterium content at the formyl carbon and the C_1 and C_2 carbons of the 1α,2α-$^2H_2$ and 1α-$^2H_1$ derivatives. Thus, the reduction of compound **5** by deuterium gas according to the method of Djerassi and Gutzwiller (1966) resulted in only partial labeling at the 1α and 2α positions. In three separate preparations, partial deuteration was consistently observed.

Table 1: Deuterium Content of Labeled Forms of ODEC

	deuterium content (%)		
labeled compound	formyl	C_1	C_2
[^{2}H]formyl	>98	0	0
1α,2α-$^2H_2$	0	44	44
1α-$^2H_1$	0	53	6

Identification of Products of ODEC Aromatization. For the identification of HTN as the aromatized product, an enzyme incubation was carried out on a large scale. A reaction mixture containing 5 nmol of P450 2B4, 5 nmol of reductase, 90 units of SOD, 1000 units of catalase, 1.25 mmol of potassium phosphate buffer, pH 7.4, 12.5 μmol of ODEC (in 125 μL of 95% ethanol), and an NADPH-regenerating system consisting of 5 μmol of NADP, 250 μmol of glucose 6-phosphate, 250 μmol of $MgCl_2$, and 5 units of glucose 6-phosphate dehydrogenase in a 25-mL final volume was incubated for 45 min at 37 °C. The mixture was extracted with two 3-mL aliquots of methylene chloride, and the combined extract was dried over anhydrous sodium sulfate and concentrated in a vacuum centrifuge at room temperature. The residue was dissolved in 20 μL of methylene chloride, and GC/MS analysis was done as above on a 3-μL aliquot of the crude product. The remaining sample was taken to dryness and dissolved in 100 μL of 40% aqueous methanol and applied to a Waters analytical C-18 reversed phase HPLC column. The column was developed with 1:1 methanol–water at a flow rate of 1.0 mL/min, and the 0.5-mL fractions collected between 10 and 12 min, which contained the purified HTN, were pooled and extracted twice with 2 mL of methylene chloride. The extract was evaporated to dryness in a vacuum centrifuge, and the residue was dissolved in 5 μL of methylene chloride and analyzed by GC/MS as above. The results are given in Figure 6. An authentic sample of the expected compound, which was obtained commercially (Aldrich), was shown in preliminary experiments to have characteristic ion masses at *m/z* 120 and 148. Examination of the crude extract of the enzyme reaction mixture showed the presence of a component having these ion masses (panel A, experiments a and b), and after purification by HPLC, the product gave a single peak in the ion chromatogram that had the fragmentation pattern shown in panel B.

For the identification of formate, five reaction mixtures similar to that used for the identification of HTN, except that the substrate was labeled with deuterium in the formyl group and all solutions were made from water that had been distilled from alkaline potassium permanganate to remove traces of formate, were pooled at the end of the incubation, acidified to pH 3.0 with 1 N sulfuric acid, and lyophilized. The frozen distillate was adjusted to pH 10 with 1 N KOH and lyophilized. The residue was dissolved in 5 mL of 95% ethanol and acidified with 80 μL of aqueous 1 N sulfuric acid, and the liberated formic acid was vacuum-transferred along with the ethanol and collected in a liquid nitrogen-cooled trap. To this solution of formic acid was added a 2-propanol solution of 4-nitrodiazobenzyl until a bright yellow color persisted for 30 min. The solvent was then removed in a vacuum centrifuge, and the residue was dissolved in chloroform and submitted to chromatography on a silica column (25 × 290 mm) with chloroform at a flow rate of 1 mL/min for 60 min followed by a linear gradient from chloroform to 100% acetone at a rate of 2%/min. The eluate was monitored at 270 nm, and fractions containing the formate ester were pooled and evaporated to dryness in a vacuum centrifuge. The residue was dissolved in 10 μL of methylene chloride, and a 2-μL aliquot was analyzed by GC/MS. The mass spectrum of the isolated product and the fragmentation pattern (Figure 6, panel C) were identical to that of an authentic sample of 4-nitrobenzyl

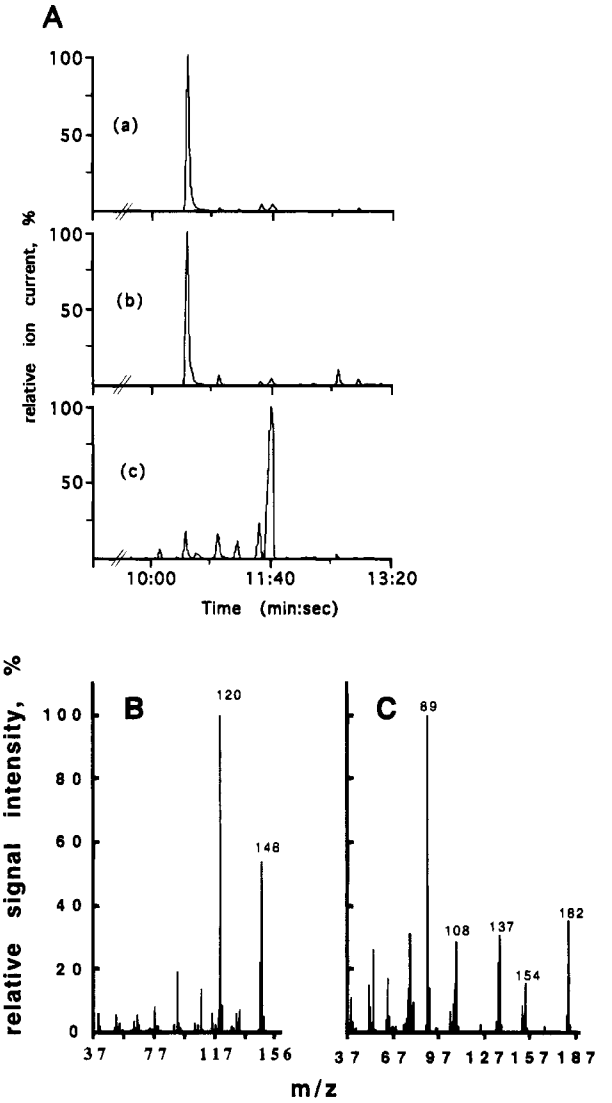

FIGURE 6: Identification of HTN and formic acid as the products of ODEC aromatization. Panel A, GC/MS analysis of enzymatically synthesized, unpurified HTN with monitoring at (a) 120 *m/z* or (b) 148 *m/z*; the total relative ion current chromatogram is shown in (c). Panel B, mass spectrum of the purified HTN. Panel C, mass spectrum of deuterated 4-nitrobenzyl formate derived from formate resulting from enzymatic cleavage of deuterioformyl-labeled ODEC.

[^{2}H]formate prepared as above from sodium [^{2}H]formate (Aldrich) (not shown).

Components Required for ODEC Aromatization. With use of the HPLC method for analysis of HTN as the aromatized product, the components required in the enzyme system were determined, as indicated in Table 2. When either P450 or the reductase was heat-inactivated or phosphatidylcholine or NADPH was omitted from the reaction mixture, little or no aromatic product was obtained. Lowering the oxygen concentration reduced the activity to about one-third of that in the complete system. Catalase and SOD were added individually and found to give a small but significant stimulation of the reaction; the reason for this effect is not known. As evidence that these enzymes do not catalyze the aromatization of ODEC, in experiments not shown HTN formation did not occur when the P450 and reductase were replaced by catalase along with glucose and glucose oxidase as an H_2O_2-generating system or by SOD along with xanthine and xanthine oxidase as a superoxide-generating system.

Table 2: Requirements for Aromatization of ODEC[a]

system	activity (nmol/min/nmol of P450)
complete	1.53 ± 0.02
P450 heat-inactivated	0.03 ± 0.02
reductase heat-inactivated	0.09 ± 0.1
NADPH omitted	0.07 ± 0.06
DLPC omitted	0.08 ± 0.06
air replaced by 99% N_2	0.53 ± 0.15
SOD added (60 units)	1.73 ± 0.11
(120 units)	1.73 ± 0.07
catalase added (120 units)	1.83 ± 0.08
(1020 units)	1.79 ± 0.09

[a] The complete system contained 0.2 nmol of P450 2B4, 0.2 nmol of reductase, and other components for the assay as described in Materials and Methods. For heat inactivation, the enzyme was boiled for 15 min prior to addition to the reaction mixture. For experiments with a reduced concentration of O_2, a septum-sealed reaction vial was placed repeatedly under a vacuum (35 mmHg) and then under 99% nitrogen for a period of 45 min prior to the addition of NADPH. The results are the mean of three experiments.

Table 3: Activity of Various Isozymes of Cytochrome P450 in the Aromatization of ODEC[a]

P450 isozyme	activity (nmol/min/nmol of P450) cytochrome b_5 absent	cytochrome b_5 present
2B4	1.10 ± 0.16	2.42 ± 0.85
1A2	0	0
3A6	<0.02	<0.02
2E1	0	0
2C3	0.14 ± 0.04	0.11 ± 0.01
2G1	0	

[a] The complete system was as described in Materials and Methods, with 0.2 nmol of a P450 isozyme and 0.2 nmol of the reductase. When present, cytochrome b_5 was added to the other enzymes before the addition of DLPC. The results are the mean of three experiments.

Isozyme Specificity and Effect of Cytochrome b_5 on the Aromatization Reaction. Table 3 shows the ability of various isozymes of rabbit P450 to effect the aromatization of ODEC. Of the six purified cytochromes examined, P450 2B4 was found to be the most active and forms 2C3 and 3A6 were approximately 10% and 1% as effective, respectively. In contrast, forms 2E1, 1A2, and 2G1 had no detectable activity. Cytochrome b_5 stimulated the activity of P450 2B4 about 2.6-fold but had no significant effect on the activities of the other isoforms examined.

Steady State Reaction Kinetics. In the assay system already described, the formation of HTN from ODEC was found to be linear for about 20 min with 0.2 nmol of P450 2B4 and 0.2 nmol of reductase present and linear with respect to the P450 concentration up to 0.2 μM. Beyond 25 min and above 0.2 μM, significant deviation from linearity was observed with respect to time and protein concentration. Accordingly, the initial velocity of the aromatization reaction was determined from experiments in which the incubation was for 20 min, with 0.2 nmol of P450 2B4 and 0.2 nmol of reductase and substrate concentrations varying from 0.05 to 1.0 mM. With these conditions, the V_{max} and K_m obtained from the double-reciprocal plot of velocity versus substrate concentration were 1.69 nmol/min/nmol of P450 and 146 μM, respectively. The effect of the molar ratio of reductase to P450 on the aromatization was also determined; a typical saturation effect was observed for the reductase with a maximal velocity of 2.37 nmol/min/nmol of P450 and a P450:reductase ratio of 0.7 at half-maximal velocity.

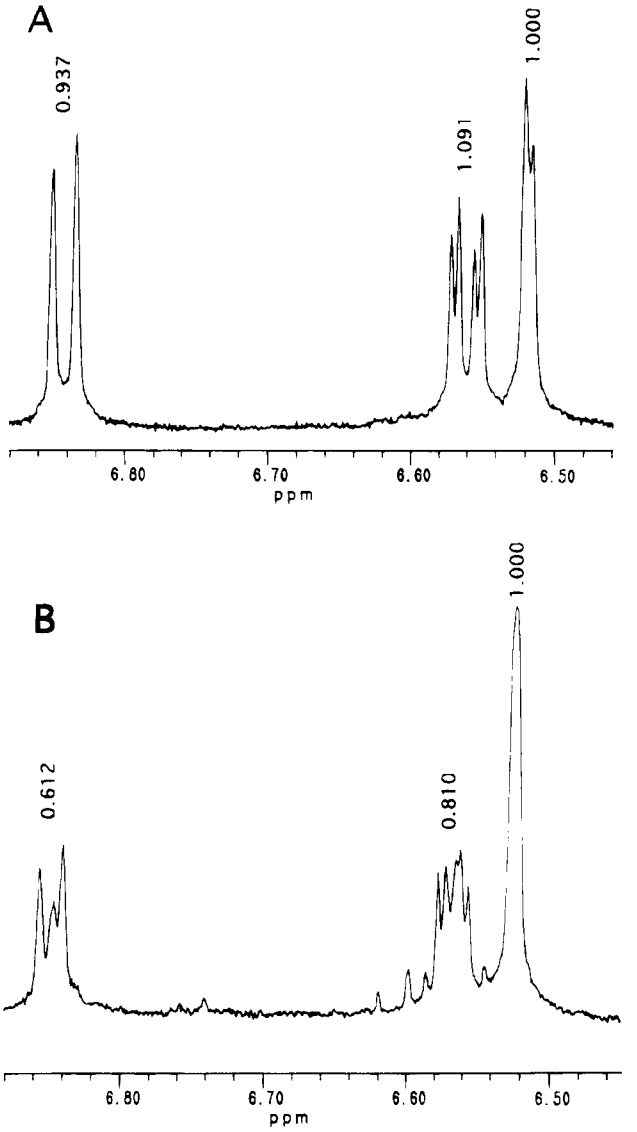

FIGURE 7: 500-MHz proton NMR spectra of HTN in the aromatic region from 6.46 to 6.88 ppm. Panel A, HTN standard. Panel B, deuterated HTN isolated from the P450 2B4-catalyzed aromatization of [1α,2α-$^2H_2$]ODEC. Numbers above the peaks are the signal intensities unitized to the signal intensity for the C_4 proton at 6.52 ppm.

Stereochemistry of Enzymatic Aromatization of ODEC. The stereochemistry of proton loss at the C_1 and C_2 positions upon aromatization of ODEC to HTN was determined by high-resolution proton NMR and mass spectroscopy. As indicated below, the NMR method is more direct, but the large amount of enzymatically derived HTN needed for this technique necessitated use of the mass spectroscopic method in some experiments. For the NMR method, HTN derived from [1α,2α-$^2H_2$]ODEC was purified by reversed phase HPLC from 60 25-mL reaction mixtures of the kind used for identification of HTN; attempts to do this with larger scale mixtures were unsuccessful. Figure 7 shows the 500-MHz proton NMR spectrum in the aromatic region for a standard of HTN and the deuterated HTN isolated from the 2B4-catalyzed aromatization of [1α,2α-$^2H_2$]ODEC. The assignments of the proton signals are C_{1H}, 6.83 ppm, C_{2H}, 6.56 ppm, and C_4H, 6.52 ppm, as given in the Sadtler

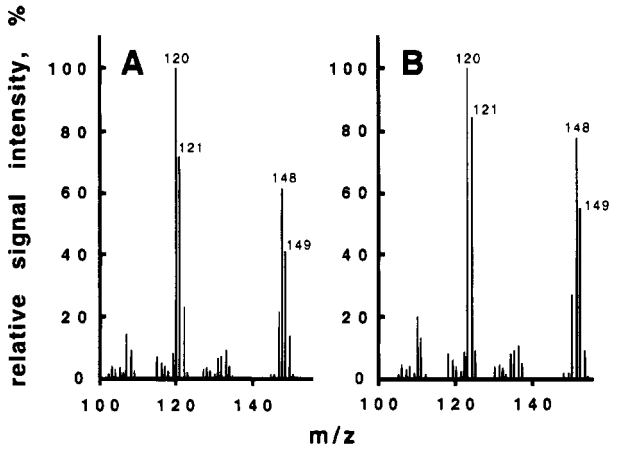

FIGURE 8: Mass spectrum of HTN generated from dideuterio ring-labeled ODEC (A) or monodeuterio ring-labeled ODEC (B).

reference work (1976). Signal intensities were integrated relative to the C_4 proton of HTN. The C_1 and C_2 proton signal intensities for deuterio-HTN are 61.2% and 81.0%, respectively, of the C_4 proton signal intensity. Stereospecific loss of the $C_{1\beta}$ proton from [1α,2α-$^2H_2$]ODEC would result in retention of the 1α-deuterium at the C_1 position of HTN. Since the C_1 position of ODEC was labeled in the amount of 44%, the NMR signal intensity would be expected to be decreased by a corresponding amount. The signal intensity for the C_1 position of HTN is 61%, indicating stereospecific loss of the $C_{1\beta}$ proton. Similarly, stereospecific loss of the $C_{2\beta}$ proton would result in retention of deuterium at the C_2 position and a corresponding decrease in the signal intensity of 44%, whereas lack of stereospecificity for loss of the C_2 proton would result in a decrease in signal intensity of only 22%. The observed signal intensity for the C_2 proton is consistent with lack of stereospecificity at the C_2 position of ODEC.

The mass spectra of HTN samples isolated by HPLC after enzymatic aromatization of the [1α,2α-$^2H_2$]ODEC and [1α-$^2H_1$]ODEC preparations are shown in Figure 8. Since the spectrum does not provide any unique ion masses that distinguish the C_1 and C_2 carbons, the deuterium content at those respective carbons could not be determined directly. Instead, on the basis of the known deuterium content at the C_1 and C_2 positions in [1α,2α-$^2H_2$]ODEC and [1α-$^2H_1$]-ODEC (Table 1), the relative ion mass intensities at *m/z* 120, 121, and 122 were predicted for the HTN produced taking into account all stereochemical possibilities at the C_1 and C_2 carbons, as shown in Table 4. The results obtained with the HTN produced from both labeled substrates are shown in Table 5. For the product derived from dideuterio-ODEC, the observed relative ion mass intensity fits best with predictions 5 or 7, whereas for the product derived from monodeuterio-ODEC the observed result is compatible with predictions 1, 2, or 5. Since the two substrates differ only in the isotopic composition at the C_1 and C_2 carbons, the prediction common to both (that is, number 5) may be taken to reflect the actual stereochemistry. Thus, we conclude that the P450 2B4-catalyzed aromatization of ODEC is stereospecific for removal of the 1β-hydrogen. The predicted isotopic abundance shown in Table 4 is based on a 100% α-facial selectivity for the reduction of compound **5** by the method of Djerassi and Gutzwiller (1966). It should be noted that Osawa and Spaeth (1971) utilized this procedure for the

Table 4: Predicted Relative Ion Mass Intensities for all Stereochemical Possibilities at the C_1 and C_2 Carbons of Dideuterio- and Monodeuterio-ODEC[a]

prediction no.	C_1	C_2	relative ion mass intensities (predicted %) [1α,2α-$^2H_2$]ODEC preparation 120	121	122	[1α-$^2H_1$]ODEC preparation 120	121	122
1	β	β	36	40	24	46	50	3
2	β	α	56	44	0	49	50	0
3	α	α	100	0	0	100	0	0
4	α	β	56	44	0	93	6	0
5	β	none	46	42	12	48	50	2
6	α	none	78	22	0	96	3	0
7	none	β	46	42	12	70	28	2
8	none	α	78	22	0	75	25	0
9	none	none	62	32	6	73	26	1

[a] The deuterium was presumed to be exclusively in the α position of ODEC. The "dideuterio preparation" contained 24% C_1,C_2 dideuterio, 20% C_1 monodeuterio, 20% C_2 monodeuterio, and 36% unlabeled compound, and the "monodeuterio preparation" contained 3% C_1,C_2 dideuterio, 47% C_1 monodeuterio, 3% C_2 monodeuterio, and 46% unlabeled compound.

Table 5: Observed Relative Ion Mass Intensities for HTN Derived from Deuterated ODEC

substrate	cyt b_5 present	relative ion mass intensity (%) 120	121	122
dideuterio-ODEC	−	51	37	11
preparation	+	51	36	12
monodeuterio-ODEC	−	51	43	2
preparation	+	51	44	2

tritiation of testosterone at the 1α and 2α positions and observed at 43:7 and 43:6 ratios, respectively, for α:β-facial selectivity. The reduction of compound **5** would be expected to show comparable facial selectivity, thus accounting for the lower than predicted isotopic abundance observed in the HTN produced from [1α,2α-$^2H_2$]ODEC (37 vs 42 predicted for mass 121) and [1α-$^2H_1$]ODEC (43 vs 51 predicted for mass 121). The results also show that cytochrome b_5, which stimulates the P450 2B4-catalyzed reaction by a factor of 2.4, has no effect on the stereochemistry of aromatization of ODEC.

DISCUSSION

The terminal reaction in the conversion of androgens to estrogens, involving substrate desaturation associated with deformylation, has been of much interest as an unusual transformation associated with steroid metabolism. However, our reports on the oxidative deformylation of a variety of xenobiotic aldehydes to olefins and formate (Vaz et al., 1991; Roberts et al., 1991) have established the generality of reactions in which P450 functions as an aldehyde lyase or oxidative deformylase−desaturase. In the present paper we have demonstrated a specialized form of the reaction in which ODEC, a xenobiotic aldehyde comparable structurally to the A and B rings of the formylandrogen intermediate, acquires an additional double bond and is thus aromatized by a purified liver microsomal P450, phenobarbital-inducible isozyme 2B4. Two critical aspects of the transformation have been established to be identical to the reaction catalyzed by steroid aromatase: the retention of the formyl hydrogen of the substrate in the formate produced and the stereochem-

istry of the reaction at the C_1 carbon. The aromatase-catalyzed reaction with androstene-3,17-dione and testosterone is known to be specific for the 1β-hydrogen, thus involving a stereospecific *cis* elimination of formate from both steroids. Similarly, the aromatization of ODEC by P450 2B4 is specific for the 1β-hydrogen. The stereospecificity for the C_2 hydrogen with placental steroid aromatase has been shown to be substrate- and temperature-dependent, with high specificity for the 2β-hydrogen with androstene-3,17-dione as substrate and a loss of specificity at higher temperature with testosterone as substrate. With ODEC at 37 °C, the reaction is stereospecific only for removal of the 1β-hydrogen and no specificity is observed for loss of the hydrogen at C_2. It should be noted from the recent interesting work of Cole and Robinson (1990c) that steroid aromatase is capable of acting on a desoxy homodiene analog of androstene-3,17-dione, thus indicating the lack of importance of stereochemistry at C_2 in the crucial deformylation reaction. In contrast to steroid aromatase, which can utilize the C_{10}-hydroxymethyl steroid as a substrate, P450 2B4 was ineffective in aromatizing the analogous bicyclic compound, 3-oxodecalin-4-ene-10-carbinol (unpublished results). Another difference is that cytochrome b_5 significantly stimulates the P450 2B4-catalyzed aromatization of ODEC but inhibits steroid aromatization (Kellis & Vickery, 1987). The effect we observed with cytochrome b_5 was only on the rate, whereas the stereochemistry of the reaction was unaffected. The reason for the stimulation by cytochrome b_5 of ODEC oxidation has not been explored, but it may be noted that in an earlier study (Pompon & Coon, 1984) the stimulation by cytochrome b_5 of reactions catalyzed by P450 2B4 was shown to be due to an increased rate of electron transfer from ferrous b_5 to the ferrous-O_2 complex.

Only limited studies have been carried out so far on the substrate specificity of the desaturation–deformylation reaction catalyzed by P450 2B4, but the information presently available indicates that branching near the aldehyde carbon is an important structural feature. Branching at the α-carbon is particularly effective, whereas branching at the β-carbon imparts slight activity to the substrate (Roberts et al., 1991). Thus, the activity of ODEC with its α-branched structure might have been predicted. A variety of physiologically occurring as well as xenobiotic aldehydes remain to be examined for deformylation by microsomal P450s. It may be noted in this connection that ketones, by formation of peroxy hemiketals, may undergo a cleavage reaction comparable to the aldehyde lyase reaction for which an enzyme-bound peroxy hemiacetal capable of undergoing concerted or sequential β-scission has been proposed (Stevenson et al., 1988; Vaz et al., 1991; Miller et al., 1991).

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