SYNTHESIS OF [2-d]ESTRADIOL, [4-d]ESTRADIOL, [2-t]ESTRADIOL AND [4-t]ESTRADIOL WITH HIGH SPECIFICITY

David J. Porubek,[a] Moses J. Namkung,[b] Mont R. Juchau,[b] and Sidney D. Nelson[a]
[a]Department of Medicinal Chemistry, School of Pharmacy and
[b]Department of Pharmacology, School of Medicine,
University of Washington, Seattle, Washington 98195

SUMMARY

17-β-Estradiol has been labeled in the 2 or 4 position with tritium or deuterium. Both 2- and 4-iodoestradiol were prepared by treatment of estradiol with N-iodosuccinimide in ethanol followed by chromatographic separation. Reductive dehalogenation of 2- and 4-iodoestradiol with tritium gas gave [2-t]estradiol and [4-t]estradiol, respectively, with specific activities of approximately 2 Ci/mmole. Greater than 99% of the radiolabel was incorporated into the target positions as determined by rehalogenation of the radiolabeled compounds. Analysis of the deuterium labeled compounds, [2-d]estradiol and [4-d]estradiol, similarly prepared by reduction with deuterium gas, revealed that isotope incorporation was high and specific.

Key Words: 17-β-Estradiol, Catecholestrogens, [2-d]Estradiol, [4-d]Estradiol, [2-t]Estradiol, [4-t]Estradiol

INTRODUCTION

Aromatic hydroxylation of estrogens at the 2 or 4 position leads to the production of catecholestrogens (Fig. 1). The catecholestrogens are recognized as major metabolites of endogenous and exogenous estrogens in humans and experimental animals (1). These substances are not inactive but exhibit a wide spectrum of biological effects including estrogenic, antiestrogenic, antitumorigenic, hormonal regulatory and various other actions (2).

Assays for direct determination of the catecholestrogens are hampered by the marked chemical instability and further biotransformation of the compounds (3). Therefore, the indirect method utilizing release of tritium from specifically labeled estrogen molecules has appeared to be the most promising

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from the viewpoint of the development of a sensitive assay (4). The accuracy of this approach is, however, highly dependent on the degree of specific labeling of the estrogen substrates. Herein, we describe a convenient route for the preparation of labeled estrogens of high specificity.

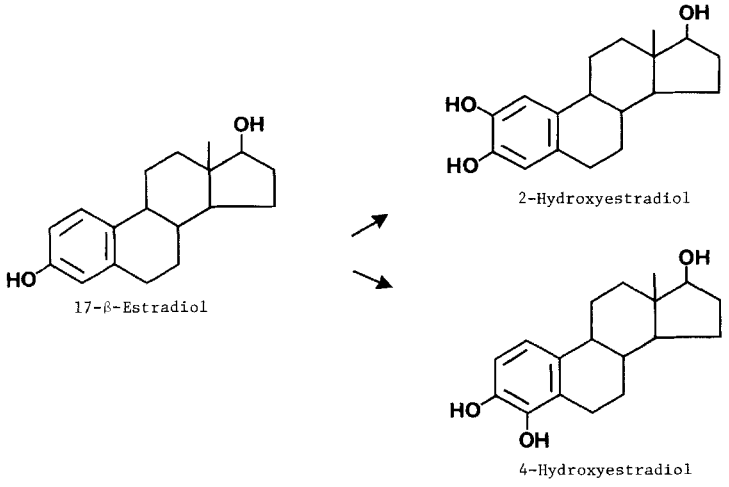

Fig. 1

EXPERIMENTAL

Instrumentation. Melting points (uncorrected) were determined on a Thomas-Hoover apparatus. Ultraviolet (UV) spectra were recorded on a Cary 219 (Varian) spectrophotometer. Infrared (IR) spectra were recorded on Perkin-Elmer 283 and Beckman IR-5A spectrometers. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian EM360A and chemical shifts are reported in parts per million relative to tetramethylsilane. Mass spectra were recorded in the EI mode (EIMS) on a VG7070H double-focusing instrument using an accelerating voltage of 4KV and an electron energy of 70eV at a nominal mass resolution of $m/\Delta m \approx 1000$ (10% valley). High resolution mass spectra were recorded at a nominal mass resolution of $m/\Delta m = 10{,}000$. For high pressure liquid chromatography, DuPont 850 and Micromeritics 7000 chromatographs were utilized. Liquid scintillation counting was performed with Beckman LS-7500 and Beckman LS-8000 instruments.

Chemicals. CH_3OD (99.5 atom % D) was purchased from Aldrich Chemical Co., Milwaukee, WI and D_2 gas (99.5 atom % D) was purchased from Matheson, Newark, CA. 17β-Estradiol, N-bromosuccinimide and N-iodosuccinimide were obtained from Sigma Chemical Co., St. Louis, MO. Spectroquality solvents and palladium on powdered charcoal (Pd/C, 5%) were obtained from Matheson, Coleman and Bell, Norwood, OH. All other utilized chemicals and solvents were reagent grade.

Synthesis of 2-and 4-iodoestradiol.

To a stirred solution of estradiol (900 mg in 55 ml absolute ethanol), an equimolar quantity of N-iodosuccinimide (3.3 mmol) was added in small portions over a period of 30 min at room temperature. The reaction mixture was stirred for an additional 30 min and then combined with 50 ml of distilled water in a separatory funnel. The steroids were extracted from the mixture with 3 separate 40 ml aliquots of ethyl ether. The ether extracts were pooled and washed twice with 25 ml of 0.1N NaOH and twice with 25 ml of distilled water. The ether was then dried over Na_2SO_4 and the solvent was removed *in vacuo* to yield a yellow oil. The oil was subjected to preparative high pressure liquid chromatography using a Whatman Partisil Mag 9 10/50 ODS-2 column. A linear water:methanol gradient (85-100% methanol) was employed over a 20 min period of elution. The flow rate was 4.0 ml min^{-1}, temperature was ambient and eluted compounds were monitored at 254 nm. Retention times for estradiol, 2-iodoestradiol and 4-iodoestradiol were 7 min, 13.6 min and 16.4 min, respectively. The diiodinated product, 2,4-di-iodoestradiol, eluted later and was discarded. Fractions containing the monoiodo steroids were collected and the solvent was allowed to evaporate, yielding 230 mg (17%) 2-iodoestradiol and 265 mg (20%) 4-iodoestradiol, both as fine white crystals. The melting point of 2-iodoestradiol was 149°C (with decomposition) and the melting point of 4-iodoestradiol was 174°C (with decomposition). Literature melting points for 2-iodoestradiol: 130°C (6), 177-178°C (dec.) (7) and 142-146°C (dec.) (8). Literature melting point for 4-iodoestradiol: 78-80°C (dec.) (7).

The proton NMR spectrum (acetone-d_6) of 2-iodoestradiol was consistent with 2-iodo substitution; isolated singlets for the protons at C-1 and C-4 of the aromatic ring were exhibited with absorptions centered respectively at 7.53 and 6.63. For 4-iodoestradiol, the NMR spectrum was consistent with the ortho proton structure, showing an AB coupling pattern for the protons at C-1 and C-2 of the aromatic ring with absorptions centered respectively at 7.16 (d, J = 8.5 Hz, H-1) and 6.73 (d, J = 8.5 Hz, H-2). EIMS for both 2- and 4-iodoestradiol showed molecular ions of m/e 398, corresponding to the molecular weight of the steroid backbone plus iodine. The IR spectrum of 2-iodoestradiol (KBr disk) showed absorptions at 3340 and 3100 cm^{-1} (OH), 2900 and 2850 cm^{-1} (CH aliphatic), 1550 and 1400 cm^{-1} (C = C aromatic) and 1190 and 1240 cm^{-1} (C-O). 4-Iodoestradiol showed absorptions at 3250 cm^{-1} (OH), 2890 and 2850 cm^{-1} (CH aliphatic), 1550 and 1400 cm^{-1} (C = C aromatic) and 1260 and 1210 cm^{-1} (C-O). UV spectra in ethanol showed absorption maxima at 290 and 297 nm for 2-iodoestradiol and at 285 and 292 nm for 4-iodoestradiol. High resolution mass spectrometry gave m/e values of 398.0730 for 2-iodoestradiol and 398.0735 for 4-iodoestradiol.

Synthesis of [2-t]estradiol and [4-t]estradiol. Tritiated estrogens were prepared by reductive dehalogenation of 2- and 4-iodoestradiol respectively. Iodinated estrogens, 45 mg (0.113 mmol) each, were provided to New England Nuclear Corp. (NEN), Boston, MA, who performed the reductions under the reaction conditions that we prescribed. The halogenated estrogens were dissolved individually in 2.0 ml methanol to which were added 25 mg of Pd/C (5%) catalyst. An atmosphere of tritium gas (25 Ci, 29 Ci/mmol) was introduced into each reaction vessel and the mixture was stirred for 10 min at room temperature. Then one equivalent of sodium thiosulfate solution was added with stirring. Labile tritium was removed in vacuo with CH_2Cl_2:MeOH (1:1). After filtration, the product was taken to dryness in vacuo and reconstituted in 10 ml of benzene:ethanol (9:1).

The tritiated estrogens were received from NEN in sealed ampoules in the benzene:ethanol solutions. Reductive dehalogenation of the iodoestrogens was found to be incomplete by mass spectrometry, therefore reduction was completed by evaporating the benzene:ethanol, redissolving the steroids in 2.0 ml methanol and blowing hydrogen gas over the solutions as they were stirred mechanically for 1 hr in the presence of 25mg of 5% Pd/C. The steroids were then filtered through Whatman No. 5 filter paper to remove the catalyst and the methanol was allowed to evaporate. The steroids were purified by HPLC using a C-18 Semi-Prep μBondapak column (Waters Associates) with an isocratic mobile phase of 66% methanol and 34% H_2O at a flow rate of 3.0 ml/min. Elution was monitored at 285 nm and fractions with retentions corresponding to estradiol were collected. Methanol was removed by overnight evaporation at room temperature and steroids were extracted twice from the water phase (approximately 2 ml) with 5 ml volumes of ethyl ether. The ether was evaporated to leave [2-t]estradiol (18 mg, 58%) and [4-t]estradiol (21 mg, 61%). Specific activities of the [2-t]estradiol and [4-t]estradiol were 2.1 and 2.0 Ci/mmol, respectively. Radiochemical yields for [2-t]estradiol and [4-t]estradiol were 0.55% and 0.61%, respectively. Radiochemical purity of the product was determined to be 98% by recrystallization from benzene of an aliquot to constant specific activity with unlabeled estradiol. Three successive recrystallizations from benzene yielded specific activities of 49.8, 50.9, and 50.6 nCi/mmole for [2-t]estradiol and 63.5, 64.4, and 65.4 nCi/mmole for [4-t]estradiol.

Locations of the tritium at C-2 and C-4 were confirmed by bromination of the compound as follows: to a solution of 50 mg [2-t]estradiol (68 μCi/mmole) in 5.0 ml dimethylsulfoxide, 40 mg of N-bromosuccinimide were added over a 10 min period. The reaction was permitted to proceed at room temperature for 60 min. Most of the dimethylsulfoxide was removed by distillation and the remaining 1.0 ml was added to 50 mls ether. The ether was washed with H_2O (3x25 mls) and then dried over Na_2SO_4. Removal of the ether <u>in vacuo</u> left a yellow

oil. The oil was subjected to preparative high pressure liquid chromatography using a C-18 Semi-Prep μBondapak column (Water Associates) with an isocratic mobile phase of 60% methanol and 40% H_2O at a flow rate of 3.0 ml/min. The eluant was monitored at 285 nm and the retention times for estradiol, 2-bromoestradiol and 4-bromoestradiol were 12 min, 20 min and 27 min, respectively. Fractions containing the steroids were collected and the solvent removed in vacuo to leave 8 mg estradiol, 19 mg 2-bromoestradiol and 21 mg 4-bromoestradiol. Halogenated steroids were recrystallized from ethanol and estradiol was recrystallized from benzene. Following three recrystallizations the specific activity of 2-bromoestradiol was 0.58 μCi/mmole while the specific activity of 4-bromoestradiol remained at 68 μCi/mmole, equal to [2-t]estradiol. Similarly, the specificity of labeling was examined for [4-t]estradiol and following three recrystallizations the specific activity of 4-bromoestradiol was 0.49μCi/mmole while the specific activity of 2-bromoestradiol remained at 91 μCi/mmole, equal to [4-t]estradiol.

Synthesis of [2-d]estradiol and [4-d]estradiol. To a stirred solution of 20 mg (0.050 mmol) 2-iodoestradiol with 5 mg Pd/C (5%) in 1.0 ml of methyl alcohol-d_1 was added an atmosphere of deuterium gas by allowing a constant stream to flow into and out of the vessel for 15 min. The mixture was then added to 15 ml cold ethyl ether in a small separatory funnel and 1.0 ml cold 0.1N HCl added which aided in precipitation of the carbon. The ether layer was filtered through a short bed of Celite and dried over sodium sulfate. Evaporation of the ether left 12 mg of product. Recrystallization from benzene yielded 8 mg (58%) [2-d]estradiol.

Synthesis of [4-d]estradiol followed the same procedure except that 4-iodoestradiol was utilized as starting material: averaged multiple scan analysis of 10 scans, 10 sec/decade, of [2-d]estradiol over a mass range of 250-300 a.m.u. showed 13.0% d_0, 84.2% d_1, and 2.7% d_2. Analysis of [4-d]estradiol showed 13.7% d_0, 83.3% d_1 and 2.9% d_2.

The proton NMR spectrum (acetone-d_6) of [2-d]estradiol exhibited isolated

singlets of equal intensity for the aromatic protons at 7.53 ppm and 6.49 ppm. [4-d]Estradiol exhibited doublets of equal intensity for the ortho aromatic protons centered at 6.57 and 7.09 ppm (J = 8.5 Hz).

RESULTS AND DISCUSSION

The accuracy of the indirect method utilizing tritium release to measure catecholestrogen formation is highly dependent on the degree of specific labeling of the estrogen substrates. This feature is of utmost importance when measuring catecholestrogen production in tissues of exceedingly low activity (e.g. brain or its substructures). Comparisons of various methodologies for the synthesis of radiolabeled estrogens demonstrated to us that unless specific procedures and great care were utilized, the tritium label would appear on other positions of the molecule (4,5). For example, our initial attempts to prepare [2-t]estradiol or [4-t]estradiol by reductive dehalogenation of the corresponding 2-bromoestradiol and 4-bromoestradiol analogs proved unsatisfactory. In particular, reduction of 2-bromoestradiol always led to some nonspecific labeling of the steroid nucleus. Therefore, we prepared the more reactive iodinated analogs for this purpose. Published procedures for the preparation of 2-iodoestradiol and 4-iodoestradiol proved to be either inadequate (6) or tedious (7). We found that iodination of estradiol by N-iodosuccinimide produced both 2-iodoestradiol and 4-iodoestradiol which could be conveniently separated by preparative high pressure liquid chromatography.

Replacement of bromine with iodine enhanced the reactivity of the halo steroids towards reductive dehalogenation. It was found that reduction of 2-iodoestradiol or 4-iodoestradiol with deuterium gas in the presence of Pd/C catalyst was complete in 15 min. The positions of the deuterium labels were examined by NMR with [2-d]estradiol exhibiting singlets of equal intensity at 6.49 ppm and 7.06 ppm, consistent with deuterium substitution at C-2. [4-d]Estradiol exhibited a pair of doublets of equal intensity centered at 6.57 ppm and 7.09 ppm (J = 8.5 Hz), consistent with deuterium substitution at

C-4. Mass spectral analysis of the deuterated estradiols confirmed the specificity of the labeling procedure.

Synthesis of the tritiated estrogens [2-t]estradiol and [4-t]estradiol was accomplished by reductive dehalogenation of the corresponding iodinated estrogens using tritium gas. The reductions were performed by New England Nuclear (NEN) under reaction conditions that we prescribed. The reduction products thus obtained were analyzed by HPLC and mass spectrometry to determine the extents of tritiation. The estrogens wre found to contain large amounts of residual halogen. Thus, it was necessary to complete the reduction in our laboratory using hydrogen gas.

Following isolation of the pure radiolabeled compounds [2-t]estradiol and [4-t]estradiol, it was necessary to show that the tritiation reaction and any subsequent manipulations yielded specifically labeled compounds. The rehalogenation reactions, followed by chromatographic separation procedures, allowed us to determine the location of the label. Bromination of [2-t]estradiol (68 μCi/mmol) yielded 2-bromoestradiol (< 0.6 μCi/mmol) and 4-bromoestradiol (68 μCi/mmol). Similarly, bromination of [4-t]estradiol (91 μCi/mmol) yielded 2-bromoestradiol (91 μCi/mmol) and 4-bromoestradiol (< 0.5 μCi/mmol). Thus, estradiol has been labeled with tritium with a high degree of selectivity at the 2 and 4 positions.

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