Synthesis of the Equine Estrogen Metabolites 2-Hydroxyequilin and 2-Hydroxyequilenin

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Equilin and equilenin make up approximately 20% of Premarin which is currently the most popular estrogen replacement therapy. Although there are numerous health benefits of estrogen replacement therapy, there are concerns over the link between estrogen replacement therapy and breast and endometrial cancer risk. One potential mechanism of estrogen carcinogenesis involves metabolism of estrogens to 2- and 4-hydroxylated catechols which are further oxidized to electrophilic/redox active *o*-quinones which have the potential to both initiate and promote the carcinogenic process. In this investigation, we have synthesized potential metabolites of equilin and equilenin, 2-hydroxyequilin and 2-hydroxyequilenin, respectively, as well as their methyl ether metabolites. These compounds were synthesized from commercially available optically pure equilin via a practical and efficient approach; five steps gave 2-methoxyequilin from which 2-hydroxyequilin was prepared by BBr_3-catalyzed demethylation in one step. Similarly, treating 2-methoxyequilin with SeO_2 followed by demethylation with BBr_3 produced 2-hydroxyequilenin. The structures of the catechols as well as those of their methoxy ethers were unambiguously characterized by one-dimensional and two-dimensional NMR experiments, including ^1H, ^{13}C, APT, COSY, HMBC, and HMQC as well as mass spectrometry.

Introduction

Currently, for most postmenopausal women who receive estrogen replacement therapy treatment in North America, Premarin (Wyeth-Ayrst) is the most prescribed estrogen formulation. The benefits of estrogen replacement therapy include relief of menopausal symptoms and prevention of osteoporosis and cardiovascular disease (*1*). In addition, there is some evidence that estrogen replacement therapy can protect women from Alzheimer's disease (*2*−*4*) and stroke (*5*). However, in recent years, there have been troubling, controversial reports concerning the slight increase in risk of developing breast and endometrial cancers, particularly for women on long-term high-dose estrogen replacement therapy (*6*−*11*). The exact mechanism(s) through which estrogens can initiate and/or promote the carcinogenic process remains both controversial and elusive. Currently, there are at least two possible mechanisms to explain the carcinogenic effects of estrogens (*11*). One theory is that estrogens are potent mitogens in hormone sensitive tissues such as the breast and endometrium. As a result, prolonged exposure of these tissues to estrogen-mediated mitogenic stimulation has long been considered an important factor in the development of hormonal carcinogenesis (*11*, *12*). Another potential mechanism is that estrogen-induced carcinogenesis could involve modification of critical cellular macromolecules by electronic/redox active quinoids which are formed from the corresponding catechol metabolite of estrogens (*12*−*14*). Recently, we have found that 4-hydroxyequilenin,[1] one of the metabolites of equilenin which is a component of Premarin, forms unusual cyclic adducts with DNA in vitro (*14*−*16*). If similar adducts are formed in vivo, which are not repaired efficiently, mutations could result, leading to initiation of the carcinogenic process in hormone sensitive tissues.

Other estrogens present in some estrogen replacement formulations, including Premarin, are equilin as well as the endogenous estrogens, estrone and 17β-estradiol. There have been very few reports about the metabolism of the equine estrogens, equilenin and equilin (*17*−*19*). Two studies (*17*, *19*) have shown that equilenin is metabolized to only one catechol, 4-hydroxyequilenin, whereas Li et al. (*18*) did report some 2-hydroxylase activity in hamster kidney microsomes. As far as equilin is concerned, Purdy et al. (*17*) reported formation of both 2-hydroxyequilin and 4-hydroxyequilin in a ratio of 2.4:1 in baboon liver microsomes. To accurately study the potential metabolism of these equine estrogens to catechols and ultimately redox active/electrophilic quinoids, it is necessary to have authentic catechol standards. The synthesis of 2-hydroxyequilenin has been reported by Ikegawa et al. (*20*), although their synthetic route was quite inefficient (15 steps) and 2-hydroxyequilenin was isolated as a racemic mixture. In this study, we have developed a new and shorter method for synthesizing 2-hydroxyequilenin (seven steps) as well as 2-hydroxyequilin (six steps) with commercially available equilin

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[1] Abbreviations: 4-hydroxyequilenin, 3,4-dihydroxy-1,3,5(10),6,8-estrapentaen-17-one; 4-hydroxyequilin, 3,4-dihydroxy-1,3,5(10),7-estrabutaen-17-one; 2-hydroxyequilenin, 2,3-dihydroxy-1,3,5(10),6,8-estrapentaen-17-one; 2-hydroxyequilin, 2,3-dihydroxy-1,3,5(10),7-estrabutaen-17-one; estrone, 3-hydroxy-1,2,5(10)-oestratrien-17-one; equilenin, 1,3,5(10),6,8-estrapentaen-3-ol-17-one; equilin, 1,3,5(10),7-estratetraen-3-ol-17-one; *o*-quinone, 3,5-cyclohexadien-1,2-dione.

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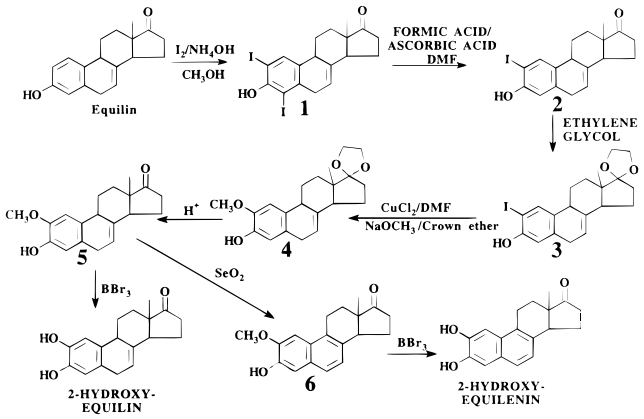
Scheme 1. Synthesis of 2-Hydroxyequilin and 2-Hydroxyequilenin



as the starting material. The synthesis of 2-hydroxyequilin has not been reported previously, although our scheme does include a modification of the procedure used to synthesize one of the intermediates, 2-methoxyequilin (*21*; Scheme 1).

Materials and Methods

Caution: *2-Hydroxyequilin and 2-hydroxyequilenin may be carcinogenic and should be handled according to NIH guidelines for the Laboratory Use of Chemical Carcinogens (22).*

Materials. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise.

Synthetic Methods. (1) 2-Methoxyequilin (5). 2-Methoxyequilin was synthesized as described in the literature (*20*; Scheme 1). Briefly, equilin (1 g) was converted into 2,4-diiodoequilin (**1**) with iodine in NH_4OH. The 4-iodo substituent in **1** was removed using formic acid/ascorbic acid, giving 2-iodoequilin (**2**). The 17-keto group in **2** was protected by reaction with ethylene glycol to produce **3**. Treatment of **3** with sodium methoxide exchanged the 2-iodo substituent for a methoxy group **4**, and acid hydrolysis followed by purification by flash chromatography on silica gel with hexane/ethyl acetate (3:2) as the eluent gave 2-methoxyequilin (**5**) as an impure solid. Further purification was accomplished using preparative TLC (silica gel) with hexane/ethyl acetate/triethylamine (10:12:3, R_f = 0.7) as the eluant to yield 150 mg of **5** (13.3% overall yield). Complete characterization was accomplished by one-dimensional (1D) and two-dimensional (2D) NMR experiments, including ^{1}H, ^{13}C, HMQC, HMBC, and COSY: ^{1}H NMR (acetone-d_6) δ 0.75 (s, 3H, 18-CH_3), 1.49 (m, 1H, H^{11}), 1.61 (m, 1H, H^{12}), 1.83 (m, 1H, H^{12}), 1.91 (m, 2H, 2 x H^{15}), 2.17 (m, 1H, H^{16}), 2.25 (m, 1H, H^{11}), 2.43 (m, 1H, H^{14}), 2.50 (m, 1H, H^{16}), 3.14 (m, 1H, H^9), 3.45 (m, 2H, 2 x H^6), 3.82 (s, 3H, OCH_3), 5.54 (m, 1H, H^7), 6.57 (s, 1H, H^4), 6.83 (s, 1H, H^1), 7.38 (bs, 1H, OH^3); ^{13}C NMR (acetone-d_6) δ 14.0 (C_{18}), 20.3 (C_{15}), 29.7 (C_6), 32.7 (C_{11}), 33.1 (C_{12}), 36.0 (C_{16}), 41.6 (C_9), 49.9 (C_{13}), 51.2 (C_{14}), 56.2 (OCH_3), 111.3 (C_1), 114.9 (C_4), 116.7 (C_7), 126.1 (C_5), 129.2 (C_{10}), 136.8 (C_8), 145.7 (C_3), 147.0 (C_2), 219.1 (C_{17}); UV (CH_3OH) 286 nm; CI-MS (positive ion, methane) *m*/*z* 299 (MH^+, 100% relative intensity).

(2) 2-Hydroxyequilin. Under an inert atmosphere, 2-methoxyequilin (15 mg, 0.053 mmol) was dissolved in anhydrous CH_2Cl_2 (4 mL) and the solution cooled to −15 °C. BBr_3 (0.5 mL of a 1 M solution in CH_2Cl_2) was added to the cooled solution, and the mixture was stirred for 9 h. Water (10 mL) was added to quench the reaction, and the solution was extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with 10% sodium bicarbonate (1 × 10 mL), 1 N HCl (2 × 10 mL), and water (1 × 10 mL) and dried over sodium sulfate. After filtration, the solvent was removed en vacuo and the residue was purified by preparative TLC (silica gel, 250 μm) with hexane/acetone/methanol (2:1:0.03) as the eluent (6 mg, 42% yield). The remaining material is 2-hydroxyequilenin which has chromatographic properties very similar to those of 2-hydroxyequilin, making purification difficult. As a result, the synthesis can only be accomplished on a small scale: ^{1}H NMR (acetone-d_6) δ 0.75 (s, 3H, 18-CH_3), 1.44 (m, 1H, H^{11}), 1.60 (m, 1H, H^{12}), 1.84 (m, 1H, H^{12}), 1.93 (m, 2H, 2 x H^{15}), 2.16 (m, 1H, H^{16}), 2.26 (m, 1H, H^{11}), 2.44 (m, 1H, H^{14}), 2.51 (m, 2H, H^{16}), 3.13 (m, 1H, H^9), 3.35 (m, 2H, 2 x H^6), 5.53 (m, 1H, H^7), 6.57 (s, 1H, ArH), 6.76 (s, 1H, ArH), 7.61 (s, 1H, exchangeable with D_2O, OH), 7.65 (s, 1H, exchangeable with D_2O, OH); UV (CH_3OH) 288 nm; CI-MS (positive ion, methane) *m*/*z* 285 (MH^+, 100% relative intensity).

(3) 2-Methoxyequilenin (6). 2-Methoxyequilin (280 mg, 0.939 mmol) was dissolved in *tert*-butyl alcohol (18 mL), followed by the addition of pyridine (0.19 mL) and selenium dioxide (128 mg, 1.2 mmol). The mixture was refluxed for 1.5 h and cooled to room temperature. The solvent was removed en vacuo, and the residue was purified by flash chromatography (silica gel) using hexane/ethyl acetate (3:2) as the eluent, giving 2-methoxyequilenin which was further purified by crystallization from methanol/acetone (110 mg, 39.5% based on 2-methoxyequilin). Complete characterization was accomplished by 1D and 2D NMR experiments, including ^{1}H, ^{13}C, HMQC, HMBC, and COSY: ^{1}H NMR ($CDCl_3$) δ 0.81 (s, 3H, CH_3), 1.92 (m, 1H, H^{12}), 2.00 (m, 1H, H^{15}), 2.21 (m, 1H, H^{12}), 2.39 (m, 1H, H^{16}), 2.54 (m, 1H, H^{15}), 2.68 (m, 1H, H^{16}), 3.18 (m, 1H, H^{14}), 3.26 (m, 2H, 2 x H^{11}), 4.03 (s, 3H, OCH_3), 5.89 (s, D_2O exchangeable, 1H, OH^3), 7.1 (d, J = 9.0 Hz, 1H, H^7), 7.2 (s, 1H, H^1), 7.26 (s, 1H, H^4), 7.55 (d, J = 9.0 Hz, 1H, H^6); ^{13}C NMR ($CDCl_3$) δ 13.1 (C_{18}), 21.8 (C_{15}), 24.3 (C_{11}), 29.1 (C_{12}), 36.6 (C_{16}), 46.8 (C_{14}), 47.5 (C_{13}), 55.8 (OCH_3), 101.4 (C_1), 110.1 (C_4), 122.2 (C_7), 125.0 (C_6), 127.3 (C_{10}), 128.5 (C_9), 129.4 (C_3), 132.4 (C_8), 144.9 (C_5), 147.5 (C_2), 220.6 (C_{17}); UV (CH_3OH) 244, 268, 278, 286, 298, 316, 330 nm; CI-MS (positive ion, methane) *m*/*z* 297 (MH^+, 100% relative intensity).

(4) 2-Hydroxyequilenin. 2-Hydroxyequilenin was synthesized using a procedure analogous to that used for 2-hydroxyequilin: ^{1}H NMR (acetone-d_6) δ 0.75 (s, 3H, CH_3), 1.80 (m, 1H, H^{12}), 1.96 (m, 1H, H^{15}), 2.15 (m, 1H, H^{12}), 2.35 (m, 1H, H^{16}), 2.61 (m, 1H, H^{15}), 2.67 (m, 1H, H^{16}), 3.17 (m, 3H, 2 x H^{11}, H^{14}), 7.1 (d, J = 9.0 Hz, 1H, H^7), 7.2 (s, 1H, H^1), 7.3 (s, 1H, H^4), 7.5 (d, J = 9.0 Hz, 1H, H^6), 8.39 (s, D_2O exchangeable, 2H, OH^2, OH^3); ^{13}C NMR (acetone-d_6) δ 13.0 (C_{18}), 22.5 (C_{15}), 24.7 (C_{11}), 29.5 (C_{12}), 36.8 (C_{16}), 47.5 (C_{14}), 47.9 (C_{13}), 106.5 (C_1), 111.2 (C_4), 122.2 (C_7), 125.5 (C_6), 128.8 (C_{10}), 129.3 (C_9), 129.5 (C_5), 133.1 (C_8), 146.2 (C_2), 147.3 (C_3), 219.0 (C_{17}); UV (CH_3OH) 242, 270, 284, 292, 304, 318, 332 nm; CI-MS (positive ion, methane) *m*/*z* 283 (MH^+, 100% relative intensity).

Instrumentation. NMR spectra were obtained with a Bruker Avance DPX300 spectrometer at 300 MHz, and CI mass spectra were obtained with a Finnigan MAT 90 magnetic sector mass spectrometer.

Results and Discussion

The major advantage of the new synthetic route shown in Scheme 1 is that we utilize the commercially available optically pure equilin as the starting material and use a synthetic route much shorter then that of Ikegawa et al. (*20*) to synthesize optically active 2-hydroxyequilin and 2-hydroxyequilenin which are potential metabolites of equilin and equilenin, respectively, in vivo. The key steps for the synthesis of 2-hydroxyequilin are the synthesis of 2-methoxyequilin **5** and its demethylation. For the synthesis of 2-methoxyequilin, we use equilin (optically pure) as the starting material; after aromatic substitution, reduction, and nucleophilic substitution of the A ring of equilin, 2-methoxyequilin was obtained with the stereochemistry of equilin. The purification of 2-methoxy-

Table 1. Reaction Conditions for the Synthesis of 4-Hydroxyequilin

chemical reagents	reaction temperature (°C)	reaction time (h)	% yield
BBr_3/CH_2ClCH_2Cl	0	5	0
BBr_3/CH_2ClCH_2Cl	25	3	0
BBr_3/CH_2ClCH_2Cl	−70	5	0
$BBr_3{\cdot}(CH_3)_2S/CH_2ClCH_2Cl$	70	2	0
$BBr_3{\cdot}(CH_3)_2S/CH_2ClCH_2Cl$	0	10	0
$(CH_3)_3SiCl/NaI/CH_3CN$	65	5	0
$BBr_3{\cdot}(CH_3)_2S/CH_2ClCH_2Cl$	−15	10	5.1
BBr_3/CH_2ClCH_2Cl	−15	9	42

equilin is problematic. Previously, it was purified by HPLC which makes it difficult to synthesize on a scale of more than a few milligrams (*20*). From analysis of the synthetic route (Scheme 1), we think one of the reasons the purification of 2-methoxyequilin is difficult is the B ring in equilin is labile and, in the presence of Cu^{2+} during nucleophilic substitution with sodium methoxide, readily aromatizes, forming 2-methoxyequilenin. 2-Methoxyequilin and 2-methoxyequilenin have very similar chromatographic properties, and separation was only achieved using hexane/ethyl acetate/triethylamine (10:12:3) as the eluant on preparative silica gel TLC plates. Using this method, we achieved a large-scale synthesis of optically pure 2-methoxyequilin (150 mg) and an overall yield of 13.3% based on equilin.

The synthesis of 2-hydroxyequilin by demethylation of 2-methoxyequilin is also very challenging due to the above-mentioned labile nature of the equilin B ring. Previously, we reported the synthesis of 4-hydroxyequilin by demethylation of 4-methoxyequilin with BBr_3 as the demethylating agent (*23*). For this synthesis, we tried a series of demethylation chemical agents using a variety of solvents and temperatures as shown in Table 1. Only BBr_3 at −15 °C for 9 h gave an acceptable yield of 4-hydroxyequilin. On the basis of the structural similarities of 2-methoxyequilin and 4-methoxyequilin, we also used these conditions to synthesize 2-hydroxyequilin. Other reaction conditions, including $(CH_3)_3SiCl/NaI/CH_3CN$ and $BBr_3(CH_3)_2S/CH_2ClCH_2Cl$, resulted in the formation of dimeric material similar to what was observed during attempts to synthesize 4-hydroxyequilin. The purification of 2-hydroxyequilin is even more challenging than that of 2-methoxyequilin again because of the presence of contaminating amounts of as much as 50% of 2-hydroxyequilenin. We experimented with a series of solvent systems and chromatographic techniques, including normal phase and reverse phase flash chromatography, preparative TLC, and preparative HPLC. We found that hexane/acetone/MeOH (2:1:0.03) on preparative silica gel TLC plates (250 μm) is the best system for purification of 2-hydroxyequilin. Complete characterization was accomplished by comparison of the ^1H and ^{13}C NMR spectra with that of 2-methoxyequilin.

2-Hydroxyequilenin was prepared by aromatization of the B ring of 2-methoxyequilin using SeO_2 followed by demethylation with BBr_3 (Scheme 1). There are several reports on the preparation of equilenin by aromatization of the B ring of equilin (*21*, *24*−*28*). In one procedure, equilin was dehydrogenated with Pd/C at elevated temperatures to form isoequilenin (*24*). Unfortunately, epimerization at the C−D ring juncture accompanied this dehydrogenation. Other reaction conditions that result in B ring aromatization of equilin derivatives include the dehydrogenation of the equilin 3-acetate with SeO_2 which yields equilenin 3-acetate in moderate yield (*25*). Another procedure involves bromination of equilin with BrCl followed by a rapid double elimination to generate equilenin (*27*). Last, epoxidation of the double bond in equilin 3-acetate with MCPBA followed by rearrangement−elimination catalyzed by Lewis acid gave equilenin in moderate yields (*21*, *26*, *28*).

To avoid some disadvantages of other aromatization methods discussed above, such as multistep procedures and epimerization of the C−D ring juncture, we chose SeO_2 as the aromatization agent for the B ring of 2-methoxyequilin to directly produce 2-methoxyequilenin. The reaction is rapid (1.5 h); the workup procedure is facile and efficient, and the yield is moderate (40%). 2-Hydroxyequilenin is obtained by demethylating 2-methoxyequilenin using BBr_3 as described for 2-hydroxyequilin. On the basis of NMR experiments, including ^1H, ^{13}C, APT, COSY, HMBC, and HMQC, the structures of 2-methoxyequilenin and 2-hydroxyequilenin were fully characterized.

In conclusion, we report here the syntheses of 2-hydroxyequilin and 2-hydroxyequilenin which are potential metabolites of the equine estrogens equilin and equilenin, respectively. The structures of the catechols as well as those of their methoxy ethers were unambiguously characterized by 1D and 2D NMR experiments, including ^1H, ^{13}C, APT, COSY, HMBC, and HMQC as well as mass spectrometry. Studies on the biological properties of these metabolites are reported in the following paper.

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